CHARACTERISATION OF IMMATURE PLATELET FRACTION IN PATIENTS WITH THROMBOCYTOPENIA PRESENTING TO A TERTIARY CARE CENTER IN INDIA

A DISSERTATION SUBMITTED IN PART FULFILLMENT OF THE REQUIREMENTS FOR THE M.D. DEGREE BRANCH III (PATHOLOGY) EXAMINATION OF THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI, TO BE HELD IN APRIL, 2017.

CERTIFICATE

This is to certify that the following dissertation bearing the title of "CHARACTERISATION OF IMMATURE PLATELET FRACTION IN PATIENTS WITH THROMBOCYTOPENIA PRESENTING TO A TERITIARY CARE CENTRE IN INDIA" is a bonafide work done by Dr. Aswathy Ashok Menon in partial fulfilment of the rules and regulations for MD Branch III (Pathology) degree examination of The Tamil Nadu Dr. M.G.R Medical University to be held in April, 2017.

Dr. Vivi Srivastava, MBBS, MD
Professor and Head of Department,
Department of General Pathology,
Christian Medical College,
Vellore, Tamil Nadu,
India.

Principal,
Christian Medical College,
Vellore, Tamil Nadu,
India.

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Dr. Joy John Mammen, MBBS, MD
Professor, Head of Department and Guide,
Department of Transfusion Medicine and Immunohematology,
Christian Medical College,
Vellore, Tamil Nadu,
India.

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Dr. Aswathy Ashok Menon,
Postgraduate Registrar,
Department of General Pathology,
Christian Medical College,
Vellore, Tamil Nadu,
India.

MD dissertation

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ABBREVIATIONS

HSCT - Hematopoietic stem cell transplant

IPF - Immature platelet fraction

ITP - Immune thrombocytopenic purpura

MDS - Myelodysplastic syndrome

MPN - Myeloproliferative neoplasm

MPV - Mean platelet volume

P-LCR - Platelet large cell ratio

PCT - Plateletcrit

PDW - Platelet distribution width

PLT - Platelet

PLT I - Impedance platelet count

PLT-F - Fluorescent platelet count

PLT-O - Optical platelet count

PLT-S - Smear platelet count

RBC - Red blood cell

CONTENTS

LITERATURE REVIEW

INTRODUCTION

Thrombocytopenia results from one or more of three processes: (1) decreased bone marrow production, (2) sequestration, usually in an enlarged spleen, and/or (3) increased platelet destruction. In evaluating the mechanism of thrombocytopenia, it is necessary to know which pathogenic mechanism is the more dominant one, decreased production or increased destruction, since treatment is vastly different for the two entities. For this purpose, bone marrow aspiration is often used. A bone marrow examination provides information about the degree of thrombopoiesis by judging the adequacy of megakaryocytes but it is subject to sampling errors, delays and subjective interpretation.

The concept of 'young' platelets or reticulated platelets has been around for over 40 years and for several years after its discovery in 1969, has been a topic of intense research, especially as evidence of it being a good indicator of thrombopoiesis mounted. Up until the late 1990's, the only way to quantify them was by fluorescence flow cytometry, which had a large repertoire of drawbacks. With the advent of automated hematology analysers, automated methods of reticulated platelet estimation also came into being, in the forefront of which is the Immature Platelet Fraction or IPF. In the recent past, there has been a lot of research done on the utility of IPF in the differential diagnosis of thrombocytopenia and it is proving to be an inexpensive, non-invasive and reliable alternative to a bone marrow examination. It is also gaining importance as a marker of platelet recovery in patients with thrombocytopenia due to chemotherapy or post hematopoietic stem cell transplant. This knowledge is being used to curtail unnecessary prophylactic platelet transfusions in these patients.

In addition to IPF, hematology analysers also provide an array of platelet parameters like Mean Platelet Volume (MPV), Plateletcrit (PCT), Platelet Large Cell Ratio (P-LCR) and Platelet Distribution Width (PDW), most of which are not yet approved for routine clinical use as there is limited data available on them.

Most studies relating to IPF have been carried out in the West and in Japan. There is no study from India that has researched the utility of Immature Platelet Fraction in the diagnosis of thrombocytopenia or its predictive value in platelet recovery in patients with a recovering marrow. The study of platelet morphology under the light microscope in cases of thrombocytopenia has also been a neglected area in the recent years and there have been no studies that have looked at platelet morphology in relation these newer platelet parameters.

This study will be the first of its kind from India to look at the utility of all the novel platelet parameters in conjunction with platelet morphology on smear in the diagnosis of thrombocytopenia and also the first from India to study patterns of IPF in patients with a recovering marrow.

PLATELETS – PRODUCTION AND MORPHOLOGY

Platelets were first discovered in 1865 by German anatomist, Max Schultze and further described in 1882 by Italian pathologist Giulio Bizzozero (1). Platelets play a major role in inflammation mediated disease processes, immunity and angiogenesis apart from its primary role in coagulation (2).

Platelets are small enucleate cytoplasmic fragments, which are formed in the bone marrow from megakaryocytes. They are produced in response to thrombopoietin, a glycoprotein that is produced in the liver, kidney and bone marrow. The receptor for thrombopoietin, c-Mpl, which is a proto-oncogene, is expressed on megakaryocytes and their precursors, platelets, endothelial cells and other hematopoietic stem cells and the levels of thrombopoietin are usually inversely proportional to the platelet count (3). Megakaryocytes mature by a process called endomitosis where in the nuclear material undergo continuous replication without accompanying cytoplasmic division. This leads to the formation of a cell with a large multilobate, polyploid nucleus with a DNA content ranging from 2n – 128n. As the megakaryocytes mature, they also develop a complex assembly of tubules and cisternae across its cytoplasm, which is in communication with the plasma membrane. This is called the Invaginated Membrane System (IMS). A mature megakaryocyte then extends pseudopod like branching projections into the bone marrow sinusoids called proplatelets. Proplatelets are composed of a long tapering tubule called the proplatelet shaft with platelet-sized swellings along it. These proplatelets serve as channels along which the platelet granules and organelles packaged in the megakaryocyte cytoplasm are transported

into platelets. The entire megakaryocyte cytoplasm is converted into proplatelets and the nucleus is finally extruded and degraded.

Individual platelets are pinched off from the tips of these proplatelets due to the shear force created as blood flows through the sinusoids (2,4). An intermediate stage of platelet development called a preplatelet has also been recently described. These are discoid, anucleate megakaryocyte cytoplasmic fragments that are 2 to 10 μm in diameter. The number of preplatelets has been found to increase following recovery after a thrombocytopenic insult. As a result, they are also being equated to large young platelets in circulation. Preplatelets can mature to form a proplatelet from which two platelets are finally released by fission (5). Around 1 x 10^{11} platelets are released per day into the circulation and each platelet has a life span of 8-10 days (3). The normal platelet count ranges from 150000/μL to 450000/μL (6).

On a peripheral blood smear stained with a Romanowsky dye, platelets appear as small discoid structures, 1.5 - 3 μm in diameter with a weakly basophilic cytoplasm and numerous azurophilic granules, which are dispersed uniformly or concentrated in the center of the cell as a granulomere. These correspond to the platelet alpha granules.

Platelets greater than 4 μm in diameter are called large platelets and those that have a diameter similar to that of an erythrocyte is called a giant platelet (7). Larger platelets are thought to be the more recently released platelets and is an indicator of thrombopoiesis (8). A study by Zeigler *et al.* reported that large platelets were seen in individuals with thrombocytopenia due to ITP whereas platelet size was normal in patients with thrombocytopenia due to bone marrow aplasia, hypersplenism or ITP

with a normal platelet count (9). Giant platelets are seen in inherited macrothrombocytopenic disorders like Bernard Soulier syndrome, MYH9 related disorders, Mediterranean macrothrombocytopenia etc. (10). Thus, the study of platelet morphology can give us a good clue towards the etiology of thrombocytopenia.

ETIOLOGIES OF THROMBOCYTOPENIA

Thrombocytopenia is defined as a platelet count below the 2.5^{th} lower percentile of the normal platelet count distribution and this value has traditionally been taken as 150000/μL (11). An international working group in 2008 came up with the proposal that a platelet count of less than 100000/μL should be used to diagnose pathologically significant thrombocytopenia as counts between 100000/μL and 150000/μL could be seen in apparently healthy individuals (12).

There are 4 main pathophysiological mechanisms of thrombocytopenia (13):

1. Decreased production from the bone marrow
2. Increased peripheral destruction
3. Increased sequestration in spleen
4. Dilution

The most important conditions that come under each group are listed out below (14)(15):

PERIPHERAL DESTRUCTION

1. Immune causes

- Immune thrombocytopenic purpura
- Post transfusion purpura
- Neonatal alloimmune thrombocytopenia

2. Non-Immune causes

- Hypersplenism due to any cause
- Disseminated Intravascular coagulation (DIC)
- Thrombotic thrombocytopenic purpura (TTP)
- Heparin induced thrombocytopenia (HIT)

DECREASED PRODUCTION

- Drug induced ex: Thiazide diuretics, ethanol, cytotoxic chemotherapy
- Viral infections - Hepatitis C virus, Human immunodeficiency virus, Cytomegalovirus
- Bacterial infections
- Myelodysplastic syndrome
- Vitamin B12 and Folic acid deficiency
- Aplastic anaemia
- Hematological malignancies (leukemia, lymphomas, myeloma)

- Metastasis from solid tumors to the bone marrow

DILUTIONAL

- Massive transfusion with non-platelet blood products
- Gestational thrombocytopenia

PSEUDO-THROMBOCYTOPENIA

- Large platelets (Due to mis-counting of platelets as red blood cells in hematology analysers)
- Anticoagulant induced (Ethylenediamenetetraacetic acid [EDTA] induced platelet aggregation).

Thrombocytopenias can also be inherited, and in this case they are classified based on their mode of inheritance or platelet size. Based on platelet size, the inherited thrombocytopenias are listed out below (10,14):

SMALL PLATELETS

- Wiskott Aldrich syndrome
- X – Linked thrombocytopenia

NORMAL PLATELET SIZE

- Congenital amegakaryocytic thrombocytopenia
- Thrombocytopenia with absent radius syndrome

LARGE PLATELETS (MACROTHROMBOCYTOPENIA)

- Bernard Soulier Syndrome
- Grey Platelet Syndrome
- Mediterranean macrothrombocytopenia
- MYH-9 related disorders (Ex: May-Heggelin anomaly, Sebastian syndrome, Fetchner syndrome)
- Paris – Trousseau syndrome

METHODS FOR COUNTING PLATELETS

The path towards developing an accurate platelet count has been one that has been riddled with many problems, owing mainly to the tiny size of these blood cells and therefore possible interference by the larger red blood cells or the difficulty in separating them out from background noise during the process of gating in automated hematology analysers. Currently, there are several approved methods by which a platelet count can be obtained. Broadly, these can be divided into manual methods and automated methods. The manual methods used in platelet counting can be either direct or indirect. Direct method involves phase contrast microscopy using the Neubaeur counting chamber method. Indirect method involves estimation of platelets on a stained peripheral smear. The automated methods that are performed in automated hematology analysers employ the principles of electrical impedance, optical light scatter and

fluorescence and flow cytometry using flourochrome labeled antibodies to specific platelet antigens. For all these methods, freshly collected, anticoagulated venous blood is the preferred sample. Blood samples obtained by capillary prick method usually have a lower platelet count due to platelet aggregation at the site of prick (6).

Phase contrast microscopy was introduced in 1953 by Brecher, Schneiderman and Cronkite(16) and in 1988, was accepted as the international reference method for the estimation of platelets by the International Council for Standardization in Hematology (ICSH). In this method, whole blood is diluted in ammonium oxalate solution whereby the red blood cells are lysed leaving the platelets and white blood cells intact. The diluted blood is then charged onto a Neubauer counting chamber (hemocytometer) and platelets are counted under a phase contrast microscope (17). This method has several drawbacks in that it is time consuming, prone to pre-analytical errors, subjective and has a high inter-observer variability with a coefficient of variation of 10%-25% (18). The advantage of this method is that it is cost effective and universally available.

The other manual method for estimation of platelets is an indirect method wherein the number of platelets are estimated by examining a peripheral smear stained with a Romanowsky dye. A field in which red blood cells are just overlapping is chosen and a count is performed here. One platelet per oil immersion field (1000x magnification) is considered equivalent to 15000 platelets/μL of blood (19)(20). The advantage of this method is that platelet morphology as well as presence of platelet aggregates which would lead to spuriously low platelet counts could be studied simultaneously.

Automated methods of blood cell counting came into widespread existence after the

discovery of the Coulter principle in 1953 by Wallace Coulter (21). The coulter principle capitalizes on the fact that blood cells are poor conductors of electricity. When cells suspended in a conducting medium are passed through an aperture across which a direct current is applied, they provide a resistance to the flow of current, which is called impedance. This resistance is picked up as a voltage peak by an ohmmeter. The amplitude of the voltage peak is proportional to the size of the cell and the number of voltage peaks corresponds to the number of cells that have passed through the aperture. The series of voltage peaks obtained is converted into a histogram with number of events on y-axis and volume on x-axis and the area under the curve of the histogram is taken as the platelet count. This method therefore is able to count as well as size the blood cells. Platelet counting using the Coulter principle started by the early 1970s and most automated hematology analysers now provide the impedance platelet count as their default platelet count. Due to the larger number of cells analysed and objectivity in analysis, there was a drastic reduction in the coefficient of variation for platelet counts. Analysers use different volume cut offs to distinguish between platelets and red blood cells as both are counted through the same channel. Here in lies the greatest disadvantage of this method as abnormalities in red cell or platelet size may lead to either being wrongly categorized. For example, microcytic or fragmented red cells may be wrongly categorized as platelets leading to a falsely high platelet count or giant platelets and platelet clumps may be mistaken for red cells leading to a spuriously low platelet count. In these situations, the analyser will generate an instrument flag and an abnormal platelet histogram and to get the true platelet count, a peripheral smear review becomes necessary (22). An advantage of this method is its low cost as compared to

other automated techniques and its high accuracy when the platelet size and red cell morphology is normal.

Another method for platelet counting is the optical method, which can be carried out with or without the addition of a fluorescent dye. This is also an automated method employed in hematology analysers. In this method, a pre-determined amount of blood is aspirated by the analyser and mixed with diluting fluid. A fluorescent dye that binds to nucleic acids may be added to this suspension depending on the type of analyser being used and this is then passed through a flow cell after hydrodynamic focusing. The stream of cells is made to flow across a light source, usually a semi-conductor diode laser or a helium-neon laser. As the laser light hits a blood cell, it is scattered in various directions. In general, forward angle light scatter gives information about size of the cell and side scatter gives information about the cell contents. The angles used in analysis vary from instrument to instrument. If a fluorescent dye is also added, then the dyes bind to the nucleic acids, mostly to ribonucleic acid which is seen in abundance in the mitochondria and ribosomes of blood cells. As the laser light hits the flourochrome, it emits fluorescence and different blood cells can be differentiated based on the intensity of side fluorescent light. These parameters are plotted on a graph where each event is depicted by a colored dot. In this way, platelets can be counted and differentiated from other blood cells based on difference in scattered light and fluorescence intensity (17). This method over comes the limitation of the impedance method of misclassifying red cells as platelets and vice versa as the fluorescent dye will not bind to red cells since they lack nucleic acids and hence red cells will not emit any fluorescence. The optical fluorescence count has also been found to be more accurate

than impedance counts in thrombocytopenic samples since it correlates better with the international reference method (23). On the other hand, for patients on chemotherapy for hematological malignancies, the impedance count has been found to be more accurate than the fluorescent count. This has been attributed to the presence of nucleic acid rich cytoplasmic fragments of blasts that take up the fluorescent dye and lead to spuriously high platelet counts (24)(25).

The most recent advancement in platelet counting is the introduction of immunological platelet counting. In 2001, the International Council for Standardization in Laboratory Hematology expert panel on cytometry and the International Society of Laboratory Hematology Task Force on Platelet Counting accepted this method as the new reference method for platelet counting. In this method, pre-diluted EDTA anticoagulated blood is mixed with platelet specific monoclonal antibodies, CD61 and CD41 and a 1:1000 dilution is made which is used for analysis. The antibodies are conjugated to fluorescein isothiocyanate (a flourochrome) and are targeted against 2 distinct epitopes on the glycoprotein IIb/IIIa complex of platelets. Initially a red blood cell count is obtained using a semi-automated impedance counter. The platelet count is obtained by a flow cytometer where the antibody bound platelets emit fluorescence as they pass across a beam of laser light. At least 1000 platelet events are analysed and the platelet count is obtained by looking at the ratio of RBCs to platelets. All hematology analysers are now calibrated using this reference method (18). The only limitation of this method is its lack of universal availability and high running costs.

PLATELET COUNTING PRINCIPLES OF ANALYSERS USED IN THIS STUDY

Three hematology analysers were used in this study and each differ slightly in the methods of platelet counting that they employ. The analytical principles used in each are as follows:

1. Beckman Coulter UniCel® DXH™ 800 (Miami, FL, USA): In this analyser, platelets are counted by impedance technology. 165 μL of blood is aspirated, diluted with an isotonic diluent and divided into two aliquots, one of which passes into the red blood cell / platelet chamber which is a common chamber for counting red cells and platelets. In here it passes through three apertures across which an electric current is applied and according to Coulter principle, voltage pulses are generated which are collected and analysed by a detector. Red blood cells (RBCs) and platelets are distinguished based on size, with particles between 2 to 20 fL being counted as platelets and particles more than 36 fL being counted as RBCs. Platelet pulses are analysed through 64 size distribution channels and a platelet volume histogram is obtained. Using the statistical least squares principles, this raw histogram is fitted to a lognormal curve and extrapolated from 0 to 70 fL to account for background noise and large platelets. The final platelet count is derived from the area under the curve. If the machine is unable to fit a curve, then system flags are generated which alerts the user to the presence of an abnormal platelet distribution due to either large platelets or platelet clumps (22,26).

2. Sysmex® XN-9000 (Sysmex, Kobe, Japan): This analyser provides three different platelet counts; Impedance based platelet count (PLT-I) and optical scatter and fluorescence based platelet counts (PLT-O and PLT-F). PLT-I is measured through a common RBC/PLT chamber using the Coulter principle. Instead of fixed thresholds, floating thresholds are used and the machine according to sample characteristics determines the lower and upper discriminators for a cell population. For platelets, the lower thresholds are between 2 – 6 fL and upper thresholds are between 12 – 30 fL. In this way, if large platelets are present, it will be included in the count, as the machine will increase its upper threshold accordingly. Here also, a platelet volume histogram is obtained but there is no fitting of the curve (17). Optical platelet count (PLT-O) is obtained in the RETIC chamber where the blood sample is mixed with a polymethine dye, which stains the reticulocyte and platelet RNA. Fluorescent platelet count (PLT-F) is obtained in a platelet specific PLT-F channel after the blood sample is stained with a platelet RNA specific fluorescent dye, oxazine. PLT-O and PLT-F are then obtained by the principle of flow cytometry as described in the previous section (27,28). PLT-F is considered to be superior to PLT-I in thrombocytopenic samples with closer concordance to the international reference method (29,30).

3. Mindray® BC-6800 (Shenzhen, China): This analyser provides two different platelet counts, Impedance and optical fluorescence based. Impedance platelet count uses coulter principle and is similar to that described above. The

fluorescent platelet count (PLT-O) is obtained in the RETIC chamber after staining with an asymmetric cyanine fluorescent dye which stains platelet and reticulocyte RNA and count is obtained by principle of flow cytometry as described above (31).

PLATELET MESSENGER RNA AND RETICULATED PLATELETS

Since platelets are without a nucleus, they are incapable of synthesizing their own Messenger RNA (mRNA), which is required for protein synthesis. Research in recent years have shown that platelets have up to 3000 – 6000 copies of mRNA which is packaged into them by the megakaryocytes. Along with mRNA, platelets also receive ribosomes, microRNAs that regulate translation of mRNAs and initiation and termination factors required for mRNA translation from the megakaryocytes. It is now believed that platelets have distinct pools of mRNA; one pool which is constitutively expressed in resting platelets and a second pool whose expression is based on the signaling by integrins. Platelet mRNA is also more stable than previously thought as it is very rich in Poly (A) tails which retard RNA degradation (32)(33).

Ingram and Coopersmith first described reticulated platelets in 1969 through an experiment they conducted on Beagles. They observed the presence of large platelets in the peripheral blood of these dogs following acute blood loss. These platelets had a high mean volume and when stained with a supra vital dye, New Methylene Blue, showed coarse punctate reticulum like structures (RNA) which were either concentrated in the center of the platelet or seen more dispersed throughout its cytoplasm. These were considered to be newly released platelets from the bone marrow which were being seen

in increased numbers following the hematological insult and they were called reticulated platelets or megathrombocytes. Studies in the 1970's also showed that reticulated platelets were more functional and that their presence had a good co-relation with the number of megakaryocytes in the bone marrow in conditions like Immune Thrombocytopenic Purpura (ITP), Systemic Lupus Erythematosus (SLE), Disseminated Intravascular Coagulation (DIC), Hypersplenism and Aplastic anaemia (34). It was also shown that platelets decrease in size in circulation as they age, further adding strength to the point that reticulated platelets were indeed newly released platelets (35,36). Shulman *et al.* in 1968 showed that non-splenectomised individuals had a longer bleeding time that splenectomised individuals at the same platelet counts, thereby suggesting that reticulated platelets were sequestered in the spleen for up to 2 days following their release from the bone marrow which probably explains the reason as to why they are seen only infrequently in the normal population (37). It has also been shown by in vivo biotinylation that reticulated platelets have a lifespan of around 24 hours (38). In light of these early investigations, reticulated platelets were thought to be more functional as well as a potential indicator of thrombopoiesis (8). An objective way of quantifying reticulated platelets came about in 1990 when Kienatz and Schmitz described the use of whole blood flow cytometry using a fluorescent dye called Thiazole orange, which bound to RNA. Analysis was done in EDTA anticoagulated blood and after gating the platelet population based on forward and side scatters, the percentage of Thiazole orange positive platelets are calculated. Reticulated platelets had more RNA and hence would bind to more Thiazole orange, thereby emitting fluorescence (39). This method had issues with standardization due to differences in incubation times,

thiazole orange concentrations and different gating strategies used, which kept changing from laboratory to laboratory. This led to the publication of very wide reference ranges (1 – 15%) and poor agreement between laboratories (40). Another main problem faced was the fluorescence labeling of nucleotides present in platelet dense granules leading to a non-specific fluorescence. In addition, this was also a highly operator dependent method and relatively expensive and thus its use was limited to research purposes (41,42).

In spite of these limitations, reticulated platelets have been widely studied as a non-invasive measure of platelet kinetics to differentiate between destructive and hypoplastic causes of thrombocytopenia. A study by Rinder *et al.* showed that an increased percentage of reticulated platelets in the setting of thrombocytopenia correlated well with increased megakaryocyte proliferation in the bone marrow whereas a decreased percentage of reticulated platelets indicated a decreased number of bone marrow megakaryocytes. The percentage of reticulated platelets were also found to be significantly higher in individuals with immune thrombocytopenia (38.6 ± 27.4%) when compared to patients on chemotherapy (7.2 ± 3.3%) and normal controls (2.9 ± 2.2%). This study also reported that 17% of cases of ITP had reticulated platelet percentage values similar to that of the control population and this was attributed to a poor thrombopoietic response (29). Salvagno *et al.* in 2005 also reported similar findings with a significantly higher percentage of reticulated platelets seen in patients with ITP, SLE, DIC, TTP and liver cirrhosis with hypersplenism when compared to normal controls (laboratory reference range taken as 6.13 ± 3.09%). The highest percentage of reticulated platelets was seen in patients with ITP (67.81 ± 18.79%) (44).

These findings were reflected in other studies as well leading to reticulated platelet percentage being considered as a cost effective and non-invasive test to replace a bone marrow examination which is the investigation of choice to diagnose a hypoplastic cause of thrombocytopenia (45,46).

Following this, studies were directed at looking at platelet kinetics in patients on chemotherapy and post hematopoietic stem cell transplant (HSCT) to see whether reticulated platelets could be used as a marker for platelet recovery. Salvagno *et al.* also studied patients with hematological malignancies on chemotherapy and noted that the percentage of reticulated platelets was similar to the control population during the myeloablative phase (10.4 ± 9.02%) and that it increased to significantly higher levels in the recovery phase (35.45 ± 6.11%) (44). Wang *et al.* studied platelet kinetics in patients on chemotherapy and noted that the percentage of reticulated platelets increased significantly during the platelet count nadir, when platelet recovery was imminent. They suggested that a reticulated platelet percentage of 17% could predict platelet recovery within 48 hours with a negative predictive value of 91% and positive predictive value of 82% (47). Chaoui *et al.* observed that a reticulated platelet count of 7% could predict platelet recovery in 4 days in patients post autologous stem cell transplant (48). Similarly, Michur *et al.* noted that the reticulated platelet percentage peak preceded platelet recovery by 3 days in patients post allogeneic stem cell transplant for various hematological malignancies (49). These studies showed that reticulated platelet percentage could be a useful tool to predict platelet recovery and thereby limit unwanted platelet transfusions in these conditions.

In addition to these, an elevated percentage of reticulated platelets in the absence of

thrombocytopenia have been found to be a useful predictor of adverse outcome in diseases associated with increased platelet turnover like pre-eclampsia, graft versus host disease (GVHD), thromboembolism associated with thrombocytosis and acute myocardial infarction (50–53). Studies have also shown that the percentage of reticulated platelets is significantly higher in patients with sickle cell anaemia in vaso-occlusive crisis, patients on hemodialysis or peritoneal dialysis and in critically ill patients with impending sepsis (54–56). These findings have helped improve the understanding of platelet kinetics in these disease processes.

AUTOMATED METHODS FOR RETICULATED PLATELET ANALYSIS

To overcome the issues faced by flow cytometric analysis of reticulated platelets, automated methods for the same were introduced in the 1990's. The first fully automated method of reticulated platelet analysis was introduced by Toa Medical (later Sysmex® Corporation) in the R-3000, a dedicated reticulocyte analyser. Here, Auramine O was used as the RNA binding fluorescent dye and a 488nm argon laser was used as the light source. Forward scattered and side fluorescent light was collected to give information on cell size and RNA content respectively and in this way, reticulated platelets were differentiated and counted (57). In the later series of Sysmex® analysers; Sysmex® XE 2100 and the Sysmex® XE 5000, reticulated platelet analysis was incorporated into the complete blood count analysers and was given the new term Immature Platelet Fraction (IPF). These instruments used a 633 nm red diode laser as the light source and an asymmetric cyanine fluorescent dye belonging to the

polymethine family of dyes, which preferentially binds RNA. IPF was measured in the Reticulocyte (RETIC) channel. Forward scattered light (cell volume information) and side fluorescent light (RNA content assessment) are analysed and a specialized computer algorithm (XE Pro-Series, Sysmex®, Japan) applies specific gates to differentiate immature from mature platelets using intensities of forward scattered light and fluorescence. This software categorizes platelets with the highest 3% fluorescence as immature platelets (58). This is then represented on a scatter plot with blue dots representing mature platelets and green dots representing immature platelets. This channel also provides the optical platelet count (PLT O). IPF is provided as a percentage of the total optical platelet count. This method had a low coefficient of variation and an initial performance evaluation had suggested a good stability of the parameter for up to 48 hours both at ambient laboratory temperatures and under refrigerated conditions (59). However, further studies highlighted a lack of stability with differences in reported stability times ranging from up to 4 hours to 24 hours with progressive increase in IPF when measured beyond these time intervals (60–62). This was attributed to the non-specific staining of the polymethine dye to the cytoplasmic fragments of white blood cells (WBCs) which had disintegrated during storage (63).

An improvement on this method was introduced in the Sysmex® XN series that is the latest technology available on the market. Here, the fluorescent dye was changed to an oxazine, which binds more specifically to platelet RNA in the rough endoplasmic reticulum and mitochondria. In addition, a dedicated chamber was added for measurement of IPF along with the fluorescent platelet count (PLT-F) (31). This improved the stability of the parameter for up to 48 hours both at room temperature and

at 4°Celsius (27).

Mindray® BC-6800 (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) is another hematology analyser that also provides an IPF. Here, analysis is carried out in the reticulocyte chamber after addition of an asymmetric cyanine fluorescent dye which binds to platelet and reticulocyte RNA (64). Forward scattered laser light and side fluorescent laser light is collected to provide an optical platelet count (PLT-O) and the IPF.

Abbott CELL-DYN Sapphire® hematology analyzer (Abbott Diagnostics, Santa Clara, California also measures reticulated platelets (retPLT) along with the assay for reticulocytes. Pre-diluted blood is mixed with a proprietary fluorescent dye called CD4K530 and is passed through a flow cell with a 488 nm green laser. Scattered light is collected at 3 different angles and fluorescent intensity is also measured. Then a specially developed algorithm is used to quantify the retPLT, which is expressed as a percentage of the total platelet count (65).

As of now, there is no universally accepted reference range for IPF as it can vary between analysers due to differences in analytical principles and between populations due to ethnic variations. Hence, laboratory specific reference ranges are to be used (31).

UTILITY OF IPF IN THE DIAGNOSIS OF THROMBOCYTOPENIA

Briggs *et al.* published the first study looking at the clinical utility of IPF in 2004. The study was done on the Sysmex® XE series and stated that the most significant increase

in IPF was seen in individuals with ITP (mean 22.3%, range 9.2 - 33.1%) and TTP (mean 17.2%, range 11.2 - 30.9%). The normal range was taken as 1.1 – 6.1%. They also noted that the IPF% fell as the platelet count improved. The test was found to be reproducible with a CV of <10% (59). A study by Abe *et al.* also had similar findings with IPF being significantly higher in ITP and in the recovery phase post chemotherapy when compared to patients with aplastic anaemia, MDS, leukemias and lymphomas. They also stated that mean platelet volume was significantly higher in ITP than in the recovery phase of chemotherapy. They suggested an IPF cut off of 7.7% to distinguish ITP from hypo-productive causes of thrombocytopenia with a sensitivity of 88% and specificity of 85.7% (66). Adley *et al.* studied IPF in patients with acute and chronic ITP and compared it to patients on chemotherapy and found out that the IPF was significantly higher in patients with ITP and more so in patients with chronic ITP. They suggested an IPF cut off of 9.4%, which could pick out patients with ITP with a sensitivity of 86.8% and specificity of 92.6%. They observed a significant negative correlation between platelet count and IPF and also suggested that IPF could be a good prognostic marker for the development of chronic ITP (67). Kickler *et al*. also concluded that IPF was significantly higher in patients with platelet destruction (ITP and DIC) and that an IPF of > 9% was 100% specific for patients with peripheral platelet destruction. In this study also the IPF of patients with a regenerating marrow, though higher than the control population, was still significantly lower than in patients with platelet destruction (68). Strauss *et al.* conducted a study in the pediatric population to differentiate between ITP and Acute lymphoblastic leukemia (ALL) based on IPF and found that IPF was significantly higher in both the patient populations, but more so in

patients with ITP. This was interesting as ALL is considered to be a hypo-productive cause of thrombocytopenia (69). Pons *et al.* and Koike *et al*. also reported similar findings and in addition showed that there was a good correlation between reticulated platelet measurement by conventional flow cytometry and IPF% (70,71). Miyazake et al studied the utility of IPF in the diagnosis of macrothrombocytopenias and reported that in MYH9 related disorders; the IPF was five times that seen in ITP (48 ± 1.9%) and twice as that seen in other macrothrombocytopenias like Bernard Soulier syndrome, αδ storage pool disease (72). All these studies had been performed on Sysmex® XE instruments.

UTILIY OF IPF IN THE PREDICTION OF PLATELET RECOVERY

Hematopoietic stem cell transplant (HSCT) is a major treatment option for many hematological diseases like leukemias, lymphomas, bone marrow failure syndromes, hemoglobinopathies etc. This can be either allogeneic (stem cells from one individual are introduced into another) or autologous (previously harvested stem cells are re-introduced into the same individual). Prior to transplant, the recipient's hemato-lymphoid system is almost completely suppressed by a preparative regimen, which can be myeloablative or non-myeloablative. This is to either completely eradicate malignant cells from the system or to prevent the recipient's immune cells from attacking the graft. So following the transplant, the recipient is usually in a phase of profound immunosuppression from which they have to recover as and when the introduced stem cells engraft and start producing progeny. Two main blood cell lines that are monitored for this are the neutrophils and platelets. According to the European Society for Blood and Marrow Transplantation, neutrophil engraftment after transplantation is defined as

an absolute neutrophil count (ANC) exceeding 500/μL for 3 consecutive days. The first of these 3 days is taken as the day of engraftment and usually occurs within 2 weeks in granulocyte colony stimulating factor (G-CSF) mobilized peripheral blood grafts. Platelet recovery is defined as the time after transplantation needed to achieve a blood platelet count exceeding 20,000/μL without transfusion support for 7 consecutive days (73,74). It is important to know the time of neutrophil and platelet engraftment, as delayed engraftment can be a sign of infection, GVHD or engraftment failure. Ongoing studies have shown that neutrophil engraftment can be predicted reliably by the immature reticulocyte fraction which is provided by some hematology analysers (75). In the same way, studies are now showing that IPF can predict platelet recovery as well in these patients since it has proved to be a reliable marker of thrombopoietic activity. Chemotherapy for various malignancies is also a condition where there is marked suppression of the hematopoietic system leading to severe thrombocytopenia. In these cases as well as in patients post HSCT; the transfusion trigger for platelets is <10,000/μL (76). Hence, an early marker for platelet recovery would be useful to prevent un-necessary prophylactic platelet transfusions in these patients.

Morkis *et al.* serially studied IPF measurements in 24 patients post autologous and 20 patients post allogeneic HSCT and suggested that an IPF cut off of >6.2% could predict platelet engraftment in up to 90% of patients and that the peak IPF% was seen before an increase in platelet count (77). Goncalo *et al.* conducted similar studies in 46 patients post allogeneic HSCT and stated that IPF could predict platelet engraftment up to 2 days before increase in platelet count (78). Takami *et al.* concluded that the IPF rose transiently >3% after around 11 days post transplant and that this rise occurred 1 day

prior to platelet engraftment (79). Van der Linden *et al.* on studies conducted on the Sysmex® XN analyser proposed an IPF cut off of 5.3% for platelet recovery within 2 days in patients post autologous HSCT. They found that the effect of platelet transfusion on IPF was unequivocal (80). This was unlike some other studies by Bat *et al.* and Briggs *et al.*, which had shown that IPF was reduced transiently by platelet transfusions. This was thought to be due to the suppression of thrombopoietin response or due to dilution of circulating immature platelets. They also had a similar finding of IPF being elevated 1 – 2 days prior to platelet count increase in post HSCT patients. Bat *et al.* also suggested that the Absolute Immature Platelet Number (AIPN) which is obtained by multiplying the IPF by the circulating platelet count / 100 is a better indicator of platelet recovery since it was not affected by platelet transfusion (81,82). Yamaoka *et al.* published that platelet recovery occurred earlier in patients undergoing HSCT with peak IPF% >10% than in those with IPF% peak values ≤ 10% and that in these patients, platelet recovery occurred within 2 – 6 days of peak IPF. Comparable findings were also seen in patients on chemotherapy for hematological malignancies (83). Another study showed similar results in the pediatric population undergoing HSCT or chemotherapy for various malignancies (84). In contrast, Have *et al.* studied serial IPF values in children on chemotherapy and said that there was no difference in platelet recovery between patients with an IPF% peak value >10% or an IPF% peak value ≤ 10%. They instead concluded that a significant increase in the AIPN of up to 0.6 x 10^9/L was seen 24 – 48 hours prior to platelet count recovery and that AIPN was not affected by platelet transfusions (85).

OTHER PLATELET PARAMETERS AND THEIR UTILITY

In addition to different types of platelet counts and IPF, hematology analysers also provide other parameters, mainly related to platelet volume. Most of these parameters show variations between hematology analysers due to differences in how platelets are counted (impedance / optical based) (86). As a result, it is necessary for laboratories to use their own analyser as well as population specific reference ranges. These other platelet parameters or platelet volume indices are listed out as follows with their means of derivation and utility in the diagnosis of platelet disorders:

1. Mean Platelet Volume (MPV): In impedance counters, it is derived from the platelet size distribution curve. In optical scatter based counters, it is calculated from the modal platelet size or from Plateletcrit (PCT%) using the formula -

MPV (fL) = PCT (%) / Platelet count (10^3/μL) (86,87). In general, a reference range of 7 – 12fL is used, but laboratory specific reference ranges are preferred (88). MPV increases progressively with storage in EDTA anticoagulated blood samples due to platelet swelling (89). MPV is inversely correlated with platelet count (90,91). Since newly released platelets are larger in size, studies were conducted to see if MPV could be an indicator of thrombopoiesis. Studies have shown that an elevated MPV can distinguish between hypoplastic and destructive causes of thrombocytopenia (92,93).

Noris et al stated that an MPV > 12.4fL (Sysmex® XE) could distinguish between inherited macrothrombocytopenias and ITP with sensitivity of 83% and specificity of 89% (94). When this cut off was used in a study with larger patient numbers, a similar specificity was obtained (95).

2. Plateletcrit (PCT): It is defined as the volume of circulating platelets in a unit volume of blood and is expressed as a percentage (6). It is provided by certain analysers and can be either directly measured as equivalent to the sum of platelet impulses which are individually detected by means of the impedance measurement principle (Sysmex® analysers) or can be derived as the product of MPV and Platelet count (Mindray® analysers) (88). It has been suggested that PCT could be a better predictor of competent hemostasis than platelet count since it incorporates both platelet count and platelet volume (86). One study has shown that PCT is significantly lower in patients with ITP when compared to patients with thrombocytopenia secondary to bone marrow failure (67).

3. Platelet Distribution Width (PDW): It is calculated from the platelet histogram as the width of the size distribution curve at the 20% level of the peak and is expressed in femtoliters (87). A few studies have shown that PDW was significantly increased in ITP when compared with hypoproductive causes of thrombocytopenia implying that it could be used as a useful adjunct in these differential diagnosis (92,96). PDW is also significantly increased in essential thrombocytosis and can be used to distinguish it from reactive thrombocytosis (86).

4. Platelet Large Cell Ratio (P-LCR): It is defined as the number of cells falling above the 12 fL threshold divided by the total number of platelets and is expressed as a percentage. A few studies have shown that P-LCR is directly proportional to IPF and that P-LCR is significantly elevated in ITP when compared to hypo-productive causes

of thrombocytopenia, thereby serving as an additional parameter to distinguish between the two etiologies (67,96,97).

AIMS

1. To characterize the immature platelet fraction (IPF) derived from the automated hematology analyzers Sysmex® XN9000 and Mindray® BC6800 in specific cohorts of thrombocytopenic patients and its comparison to normal controls at a tertiary care center in India.

2. To serially monitor IPF in patients undergoing hematopoietic stem cell transplantation and correlate the trends in IPF to the timing of platelet recovery.

OBJECTIVES

1. To determine the IPF and other platelet indices like Mean platelet volume, Plateletcrit, Platelet distribution width and Platelet large cell ratio in specific cohorts of patients with thrombocytopenia using the Sysmex® XN9000 and Mindray® BC 6800 automated hematology analyzers.

2. To determine whether these platelet indices can be used to reliably distinguish between different causes of thrombocytopenia.

3. To correlate these indices with morphological features of platelets on a stained peripheral smear.

4. To analyze the trends in IPF in patients undergoing hematopoietic stem cell transplantation and to see whether it can be a reliable predictor of platelet recovery.

5. To get the normal reference range for IPF% in adults and children in the laboratory.

MATERIALS AND METHODS

This study was conducted in the Department of Transfusion Medicine and Immunohematology in conjunction with the Department of Clinical Hematology, Christian Medical College, Vellore.

The study was approved by our Institutional Review Board.

The study duration lasted from April 2015 to December 2015.

In the initial study protocol submitted (annexure 2) we had also included two additional disease groups for IPF trend analysis in a recovering marrow i.e. patients with Aplastic anaemia on Anti Thymocyte Globulin therapy and patients with Acute myeloid leukemia on chemotherapy. However, during the duration of study, the number of patients that fulfilled these criteria were very small (N=7 and N=9 respectively) and hence the data that we have from these patient groups were not analysed.

I. <u>Characterization of platelet indices in patients with thrombocytopenia and their correlation with platelet morphology on peripheral smear.</u>

Patients with thrombocytopenia usually present to the Hematology outpatient department (OPD) with bleeding symptoms or are incidentally picked up as part of a routine evaluation. In our hospital, every patient presenting for the first time to the Hematology OPD are subject to a complete blood count evaluation if deemed necessary by the clinician. These samples are collected by an evacuated tube system and analysed within 4 hours of collection in any one of the three automated hematology analysers (i.e. UniCel® DxH 800, Sysmex XN® 9000 and Mindray BC® 6800) in place in our laboratory. Peripheral smears are automatically made for

samples with platelet counts less than 100000/μL using the using UniCel® DxH Slidemaker Stainer according to our laboratories' smear review criteria. A platelet count of less than 100000/μL is considered to be significant and the next step usually entails a bone marrow examination (trephine biopsy and aspirate smears) to assess the adequacy of megakaryocytes and the presence/absence of primary bone marrow pathology. Additional investigations if needed are carried out and a final diagnosis is then given with treatment being initiated accordingly.

Inclusion criteria:

- All patients presenting for the first time to the Clinical Hematology OPD in our hospital with an initial platelet count of less than 100000/μL were enrolled in the study irrespective of whether or not they had had previous treatment for their condition from another hospital. This stand was taken since our institution is a tertiary referral care center.

Exclusion criteria:

- Patients who had presented to the Hematology OPD for similar or other hematological complaints prior to the start of the study period.
- Cases in which peripheral smear slides were not available for review.

Patient demographics, diagnoses, bone marrow trephine and aspirate reports were retrieved from the clinical workstation database. Peripheral smear slides were retrieved from the slide archival files. All patient diagnoses and classifications were

made based on established international guidelines. The presence or absence and adequacy of bone marrow megakaryocytes were recorded from bone marrow trephine or aspirate reports in which megakaryocytes had been graded as adequate, increased, decreased, absent and with or without dysplastic changes based on accepted guidelines.

The patients who were finally included in the study encompassed all the patient groups mentioned in our approved study protocol. Patients diagnosed with chronic liver disease from Gastroenterology OPD (as mentioned in our protocol) were not included in the study as we encountered a good number of patients with thrombocytopenia due to chronic liver disease or hypersplenism presenting to the Hematology OPD as well.

Sample collection and analysis:

Blood samples were collected as part of routine clinical workup into Vacutainer tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria), anticoagulated with K_2EDTA (Ethylenediamenetetraacetic acid dipottasium salt). The blood left over in the tubes after initial routine complete blood count was used for this study purpose. For this second run, the samples were analysed within 24 hours of collection with blood samples being stored at 22-24° Celsius (ambient laboratory temperature). Samples were analysed in two hematology analysers, Sysmex XN® 9000 (in the DIFF + PLT-F mode) and Mindray BC® 6800 (in the CDNR mode).

The impedance platelet counts (PLT-I), fluorescent platelet counts (PLT-F, in Sysmex), optical platelet counts (PLT-O, in Mindray) and other platelet parameters like MPV, PCT, PDW and P-LCR were retrieved from the system database and tabulated in an MS Excel sheet. In those situations where an MPV was not provided by either of these two analysers, patient results from the initial sample run were retrieved. If the initial run was carried out on the UniCel® DxH 800 Coulter hematology analyser, an MPV would invariably have been provided and this value was taken for analysis.

Peripheral smear examination:

Modified Wright-Giemsa stained peripheral blood smears were made for all the study samples within 4 hours of sample collection using UniCel® DxH Slidemaker Stainer (Annexure 4). These were evaluated for the following:

1. Platelet estimate on peripheral smear (Annexure 5) - One platelet per oil immersion field in an area where the red blood cells are just overlapping was equated to 15000 platelets/μL of blood. 20 such fields were counted and the average platelet count was calculated.
2. Platelet size – A maximum of 100 and a minimum of 5 platelets (based on the baseline platelet count) were analysed on each peripheral smear and they were sized into the following groups:
   (a) Normal – Normal sized platelets (1.5 - 3μm in diameter) .

(b) Large – Platelet size slightly smaller than that of a red blood cell (> 4μm in diameter) (7) or slightly smaller than the nucleus of a small lymphocyte, keeping the nucleus of a small lymphocyte as an index.

(c) Giant – Platelet size larger than that of a red blood cell.

3. Platelet cytoplasm – The cytoplasm of all the sized platelets were categorized according to their colour as follows:

   (a) Grey

   (b) Bluish grey

   (c) Blue

II. Trends of IPF in patients undergoing hematopoietic stem cell transplantation and its relationship to platelet recovery and platelet transfusion requirements.

Inclusion criteria:

- Patients diagnosed with Aplastic Anaemia or Thalassemia and undergoing allogeneic hematopoietic stem cell transplantation.
- Patients diagnosed with a Lymphoma or Multiple Myeloma and undergoing autologous hematopoietic stem cell transplantation.

Patient demographics, diagnoses, conditioning regimens used, total number of platelet transfusions and complications during transplant were retrieved from the clinical workstation and transplant summaries.

Sample collection and analysis:

As part of routine protocol, blood samples are collected from these patients at the bedside through an evacuated tube system every day or every alternate day and sent to our laboratory for complete blood counts. The sample left over after the initial analysis was then run again within 24 hours of storage at ambient laboratory temperature on Sysmex XN®9000 (in the PLT-F mode) and Mindray BC®6800 (in the CDNR mode). PLT-F/PLT-O counts and IPF values were retrieved from the system database and tabulated in an MS Excel sheet. Serial samples from each patient were analysed in this way until platelet recovery (defined as the third day of an unsupported platelet count of more than 20000/μL).

We have collected serial samples rather than timed samples as mentioned in our protocol (annexure 2) since we did not want to interfere with routine practices for peripheral blood count monitoring post HSCT that are in place in our institution.

III. Reference range calculation for platelet indices

Since we did not have laboratory and analyser specific reference ranges for MPV, IPF, PCT, P-LCR and PDW, it was decided to calculate the reference ranges for the same in adults and children (<18 years).

For reference range calculation in adults, blood samples from 248 voluntary blood donors presenting to our blood bank were run within 4 hours of collection in Sysmex and Mindray analysers. For reference range calculation in children, blood samples from 27 healthy children being worked up as prospective stem cell donors and presenting to our HLA testing laboratory were run within 4 hours of collection in the

Sysmex analyser. All the samples had a normal platelet distribution histogram with no system / suspect flags.

The Kolmogorov-Smirnov test for normality was performed on all the platelet parameters and they were all found to have a p value <0.05 which is indicative of a non- normal distribution. Hence, reference ranges for MPV, IPF, PDW, P-LCR, PCT and PDW were calculated using 5^{th} and 95^{th} percentiles as cut offs.

IV. Assessment of stability of platelet counts and platelet indices

Since literature has given contradicting information on stability of platelet parameters on the same as well as different analysers, we decided to carry out stability tests of these parameters on the XN as well as the Mindray platforms before the start of the study. This was carried out as follows:

Paired blood samples were collected from 23 healthy, voluntary blood donors into 4ml vacutainer tubes with dipottasium ethylenediamenetetraacetic acid as an anticoagulant. One set was stored at 22-24°C (ambient lab temperature) & other at 2-8°C (specimen storage refrigerator). Platelet parameters were measured every 4^{th} hour till 24 hours, then at 32^{nd} and 48th hours on both analysers. Results were tabulated in an Excel sheet and analysed using SAS 9.4 software.

This data was also used to study the agreement between the two analysers using Bland-Altman agreement plots.

ANALYTICAL PRINCIPLES

Sysmex® XN 9000

PLT-I is measured in the RBC/PLT channel using the principle of electrical impedance after treating the aspirated blood with a diluent, and the platelets are counted and sized based on the number and peak of voltage pulses obtained. The final platelet count is given from an algorithm that derives the data from the area under the curve of the platelet histogram. PLT-O is measured in the RETIC channel after staining with a nucleic acid binding dye called Polymethine which is an asymmetric cyanine fluorescent dye. PLT-F and IPF are measured in the PLT-F channel after treating the blood with a diluent (Cell pack DCL®) and staining with a proprietary dye, which belongs to the class of Oxazine dyes. This dye is said to be more specific in binding to the nucleic acids within the mitochondria and ribosomes of platelets. The platelet count here is obtained from the scatter plot of Forward Scatter against Side Fluorescence. From the fluorescent platelet count, the immature platelet fraction is derived using a gating mechanism, which analyses platelets above a certain fluorescent threshold and gives the IPF as a percentage of the total fluorescent platelet count.

The MPV, PCT, PDW and P-LCR are obtained from the platelet histogram, which is obtained from the RBC/PLT chamber.

Mindray® BC 6800

PLT-I, MPV, PCT, PDW and P-LCR follow similar measurement principles as detailed above. PLT-O is measured in the RETIC chamber after staining with an asymmetric cyanine fluorescent dye and the IPF is derived as a percentage of the total optical platelet count.

STATISTICAL ANALYSIS

Statistical significances between different subgroups of patients with thrombocytopenia were calculated using independent two-sample t-tests or Mann-Whitney U-tests depending on the normality of distribution.

P-value of less than 0.05 was considered to be statistically significant.

Software STATA version 11.0 was used for statistical analysis.

ILLUSTRATIONS

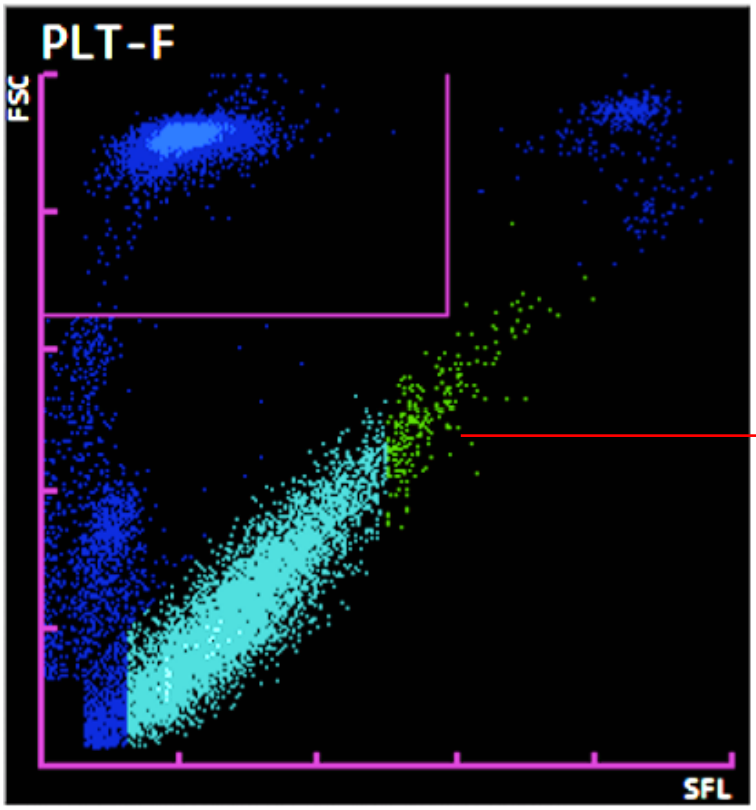


Figure 1: Normal PLT-F scatter plot on Sysmex XN 9000 in an individual with normal IPF.

Legend: PLT-F - Fluorescent platelet; FSC - Forward scatter; SFL - Side fluorescence.

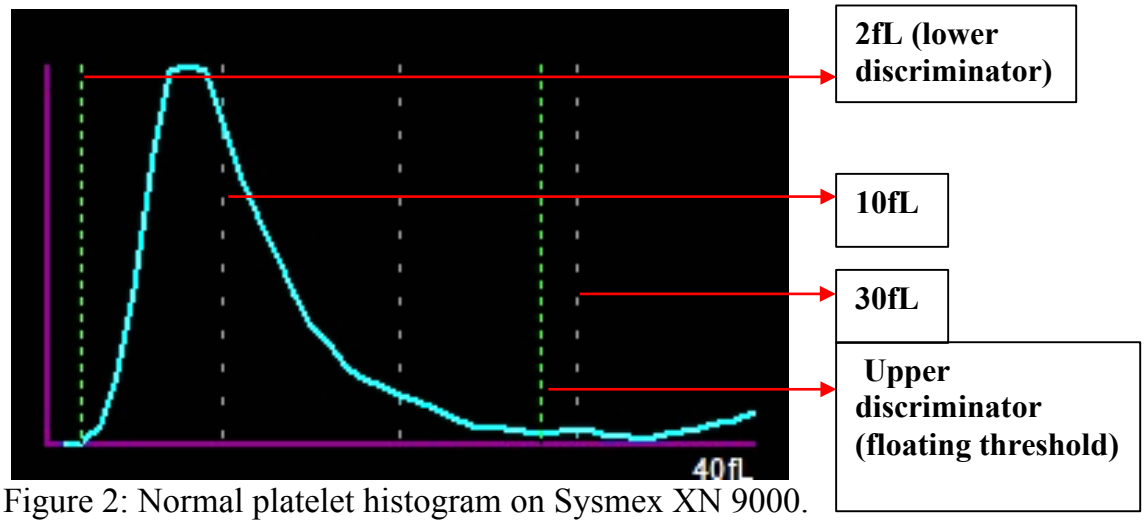


Figure 2: Normal platelet histogram on Sysmex XN 9000.

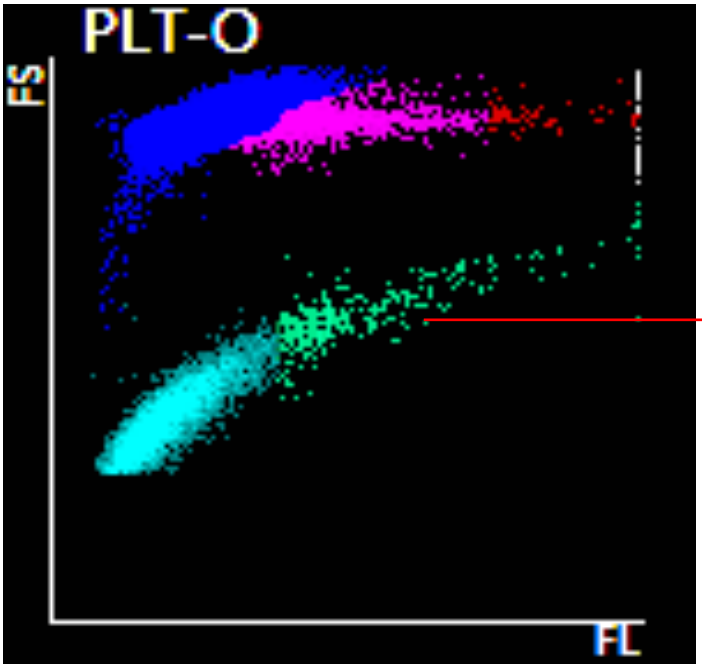


Figure 3. Normal PLT-O scatter plot on Mindray BC 6800 in an individual with normal IPF.

Legend: PLT-O - Optical platelet; FS - Forward scatter; SFL - Side fluorescence.

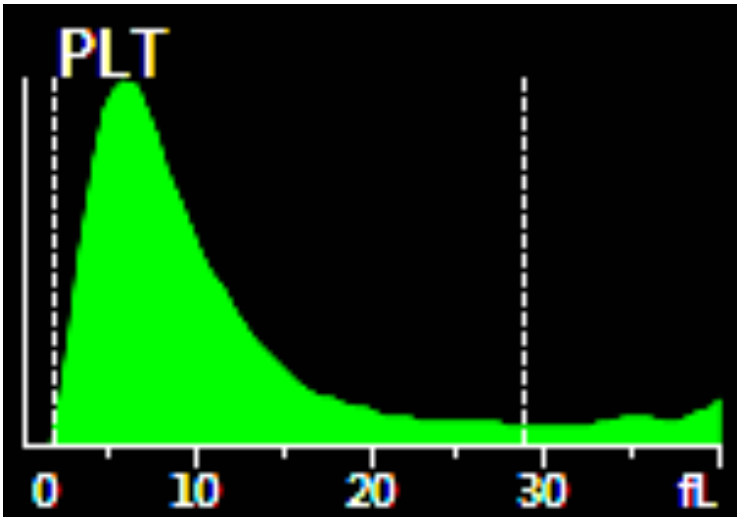


Figure 4. Normal platelet histogram on Mindray BC 6800.

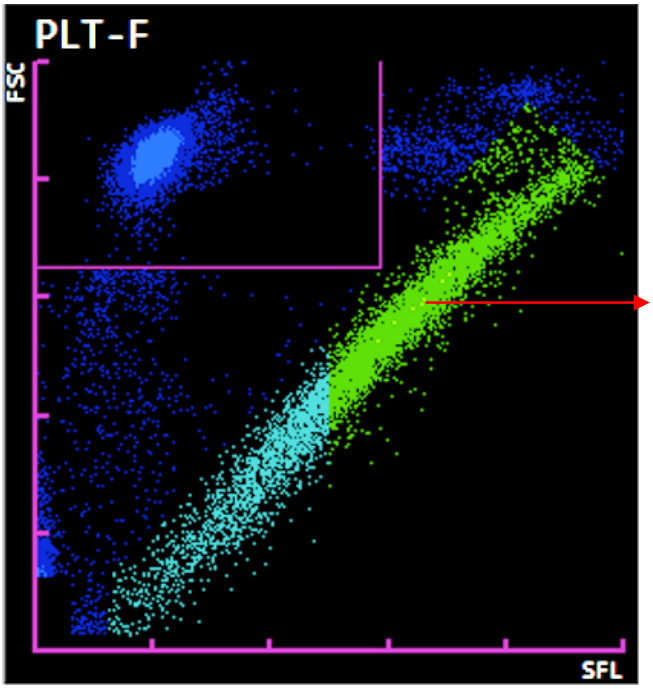


Figure 5. PLT-F scatter plot of a patient with high IPF (IPF = 71%).

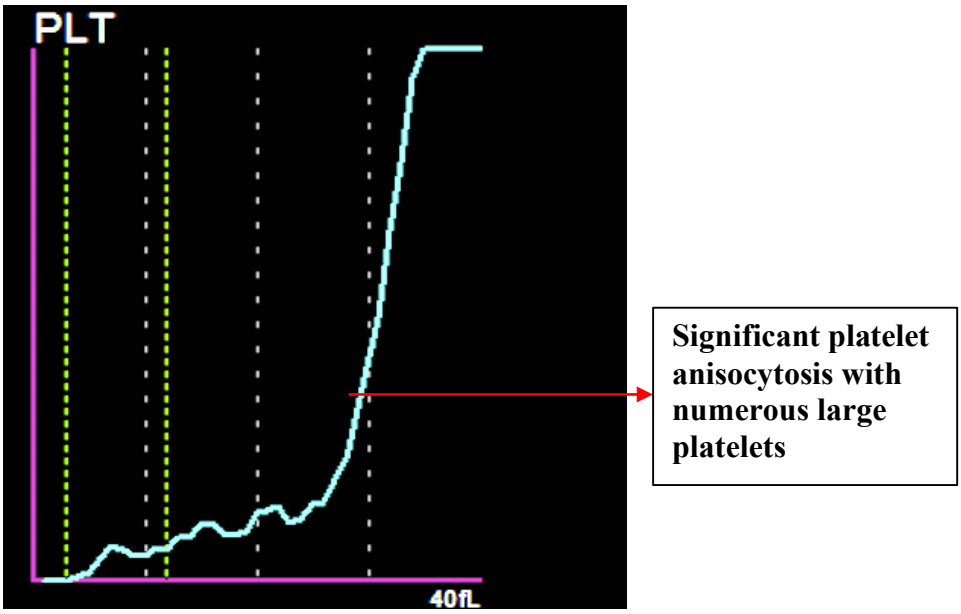


Figure 6. Platelet histogram of the same patient as above reflecting the presence of giant platelets.

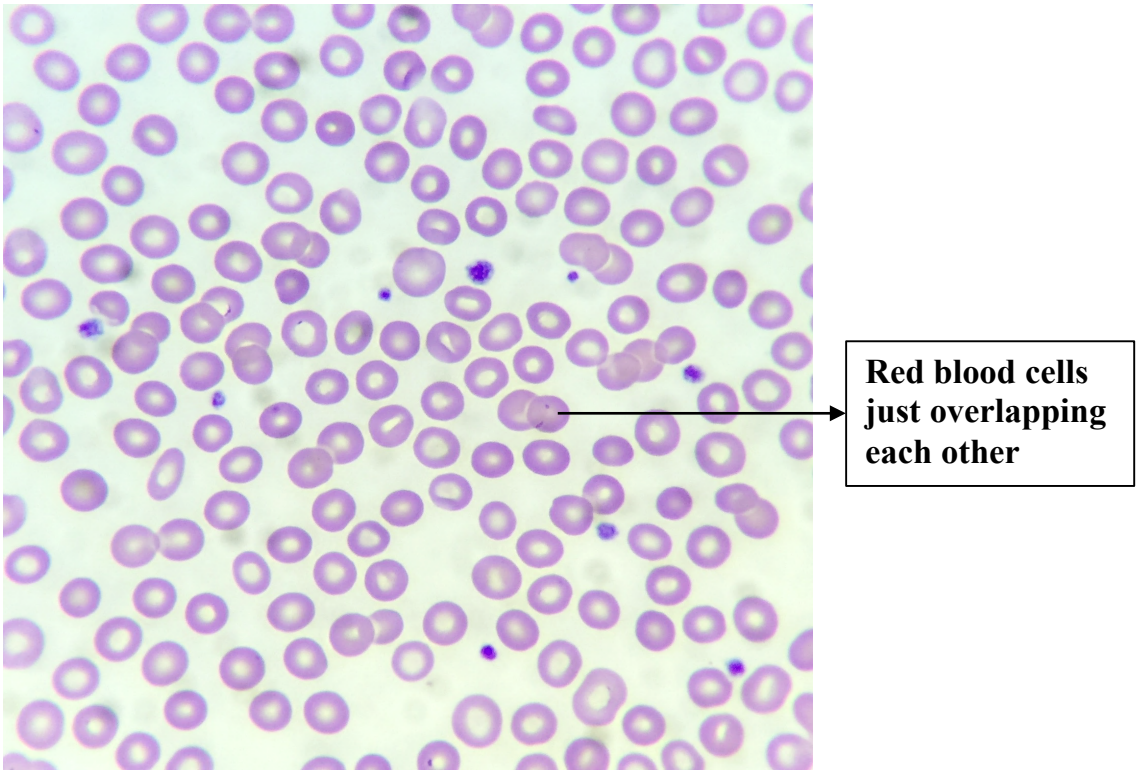


Figure 7. Normal sized platelets in a representative field used for platelet counting on peripheral smear (Modified Wright-Giemsa stain, Oil immersion - 1000X magnification).

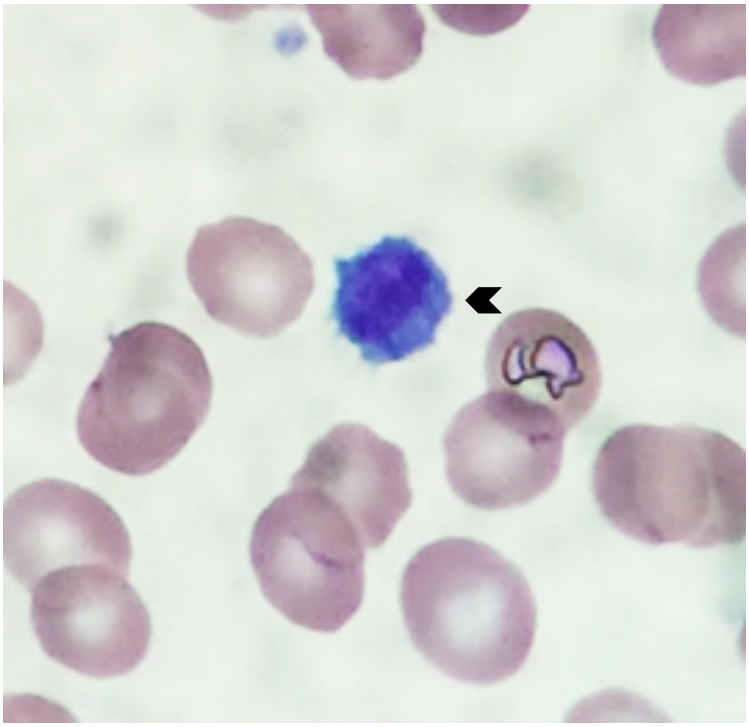


Figure 8. A large platelet [indicated by black arrow head]; equal to the size of a normocytic red blood cell and with deep blue cytoplasm - Immature platelet. (Modified Wright-Giemsa stain, Oil immersion - 1000X magnification).

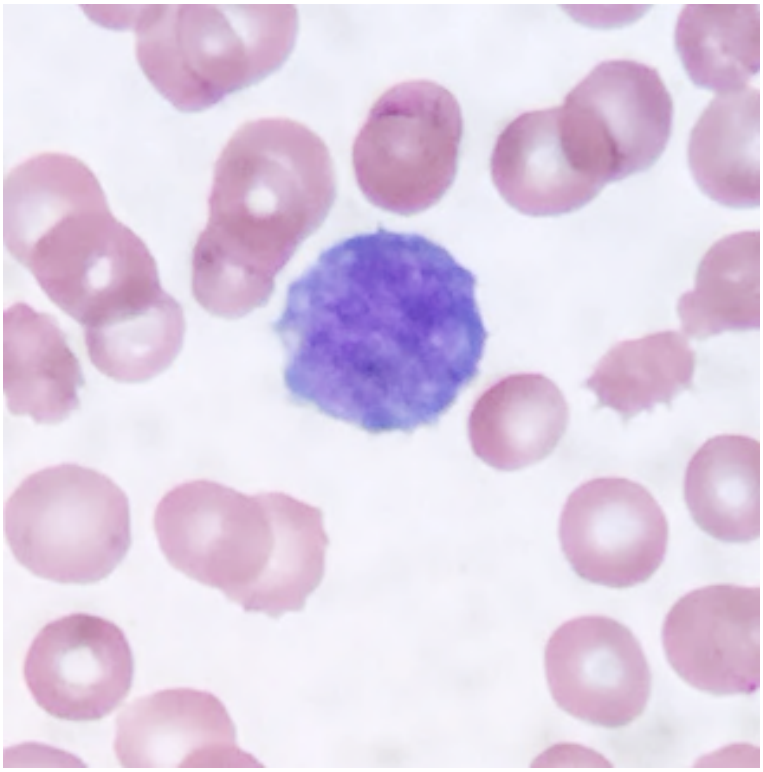


Figure 9. A giant platelet (larger than the size of a normocytic red blood cell) with deep blue cytoplasm (Modified Wright-Giemsa stain, Oil immersion - 1000X magnification).

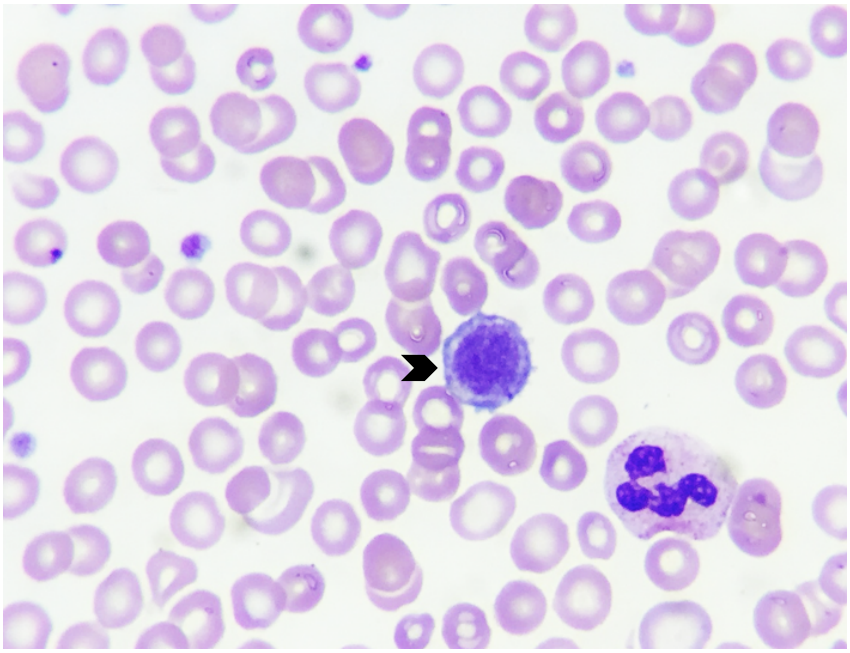


Figure 10. A giant platelet with blue cytoplasm [indicated by black arrow head] in a patient with ethnic macrothrombocytopenia and other normal platelets with grey-blue cytoplasm. (Modified Wright-Giemsa stain, Oil immersion - 1000X magnification).

RESULTS

I. REFERENCE INTERVALS FOR PLATELET PARAMETERS

Several quantitative platelet parameters are now available on hematology analyzers. The following are directly measured; Platelet count (Impedance, Optical/ Fluorescence) and Immature platelet fraction and the remaining i.e. Mean platelet volume, Platelet distribution width, Plateletcrit and Platelet large cell ratio are derived from other primary modalities. We have determined reference intervals for these using the normal population that we studied for adults and children. Since all parameters had a non-parametric distribution, reference intervals were calculated using the 5^{th} and 95^{th} percentiles (Table 1).

Table 1. The normal reference intervals obtained for our population on Sysmex XN9000 and Mindray BC6800.

PARAMETER	SYSMEX	MINDRAY
	Median (5^{th} – 95^{th} percentile)	Median (5^{th} - 95^{th} percentile)
MPV (fL)	10.6 (9.5 - 12.1)	9.5 (8.3-11.2)
IPF (%)	2.5 (1.1 - 6.1)	4 (1.6-8.9)
PDW (fL)	12.6 (10.4-16.3)	15.9 (15.4-16.5)
PCT (%)	0.3 (0.22-0.42)	0.28 (0.20-0.39)
P-LCR (%)	30.1 (20.7-42.5)	23 (14.9-35.4)

Since there is a high prevalence of constitutional macrothrombocytopenia in individuals from North Eastern India who come to our institution, we calculated a separate set of reference intervals for them using data from 131 blood donors coming from these areas (Table 2).

Table 2. Reference intervals obtained for North East Indian population on Sysmex XN 9000.

Parameter	Reference intervals (5^{th}- 95^{th} percentile)
MPV (fL)	10.3 – 14.9
IPF (%)	2.1 - 34.8
PDW (fL)	12.0 - 24.1
PCT (%)	0.19 - 0.37
P-LCR (%)	27.8 - 64.2

Table 3. Reference intervals obtained for children on Sysmex XN9000.

Parameter	Reference intervals (5^{th}- 95^{th} percentile)
MPV (fL)	8.6 – 11.3
IPF (%)	0.4 – 3.1
PDW (fL)	7.9 – 14.8
PCT (%)	0.22 – 0.47
P-LCR (%)	13.4 - 36

The difference between adult and child populations was probably due to the smaller sample size used in the reference interval calculation for children (N=27) when compared to the sample size of 248 in the reference interval calculation of the adult population.

II. AGREEMENT BETWEEN THE TWO ANALYSERS

As a part of the standardization process, we performed Altman-Bland agreement statistics on the IPF data obtained from analysis on the Sysmex and Mindray platforms.

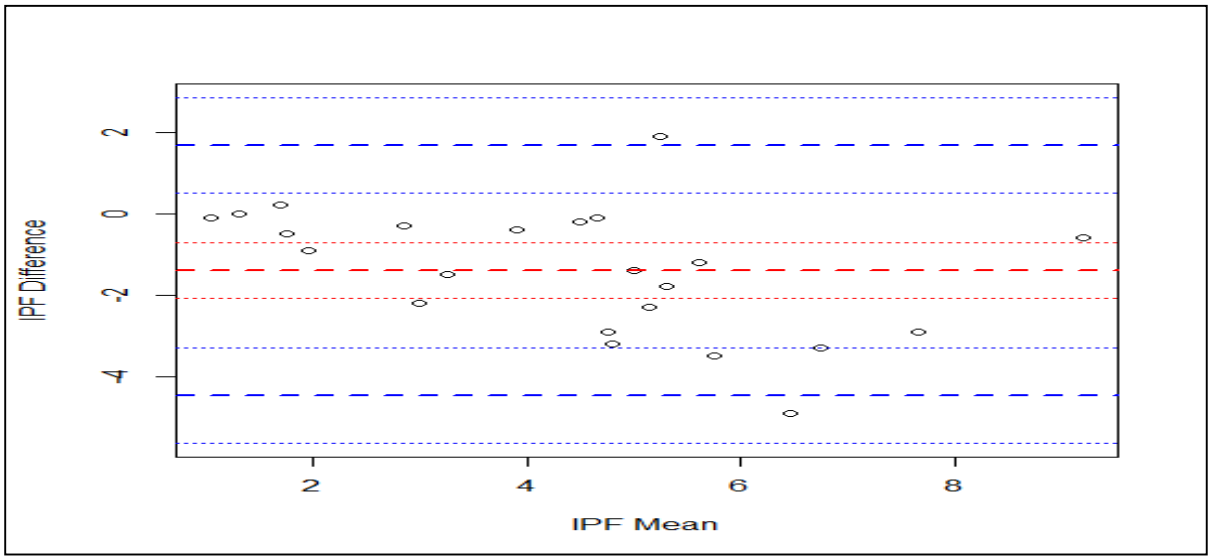


Figure 1. Altman-Bland agreement plot for IPF (Immature platelet fraction) between the Sysmex and Mindray analysers.

The above figure shows that majority of the data points fall within the 95% confidence intervals. There was no systematic bias observed between the two analysers. The Intra-class correlation coefficient was found to be 0.92 (95% CI: 0.81-0.97) which suggested that there was a good agreement between the two analysers.

III. STABILITY OF PLATELET PARAMETERS ON THE TWO ANALYSERS

Given the fact that clinical laboratories have different workflows based on where samples are sourced from and the need for add-on testing on samples already in the laboratory, stability of the measured parameter is an important consideration. We evaluated the stability of platelet parameters by timed repetitive analysis of samples stored at different temperature conditions up to 24 hours.

Table 4. Time (in hours) within which samples have to be analysed on Sysmex and Mindray analysers to get reliable results when stored at two different temperatures.

PARAMETER	SYSMEX (IN HOURS)		MINDRAY (IN HOURS)	
	22-24^{o}C	2-8^{o}C	22-24^{o}C	2-8^{o}C
IPF (%)	48	48	8	4
MPV (fL)	24	4	24	4
PLT F (x 10^9/L)	32	48	Not provided	Not provided
PLT I (x 10^9/L)	48	48	48	48
PLT O (x 10^9/L)	48	48	48	48
P-LCR (%)	32	0	32	0
PCT (%)	48	24	48	24
PDW (fL)	24	4	48	48

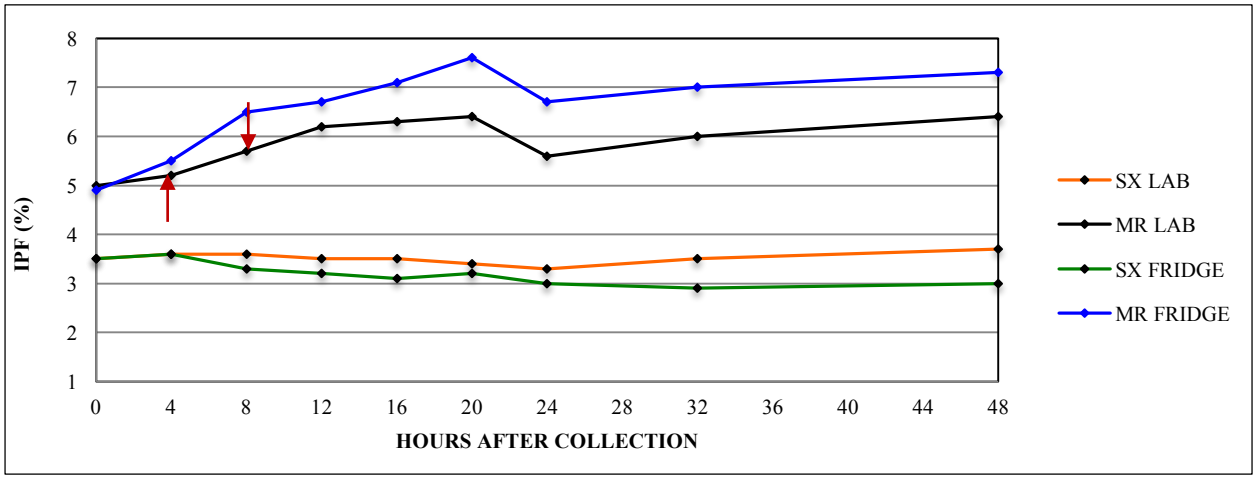


Figure 2. Serial IPF measurements over time in Sysmex (SX) and Mindray (MR) analysers in samples stored at 22-24°C (LAB) and 2-8°C (FRIDGE) temperatures.

The IPF in the Sysmex analyser was stable up to 48 hours at both the storage conditions (Table 4) whereas the IPF obtained in the Mindray analyser was stable only up to 8 hours when stored at 22-24°C and up to 4 hours when stored at 2-8°C (indicated by red arrows in figure 2), beyond which, there was a significant rise in IPF% above the true value.

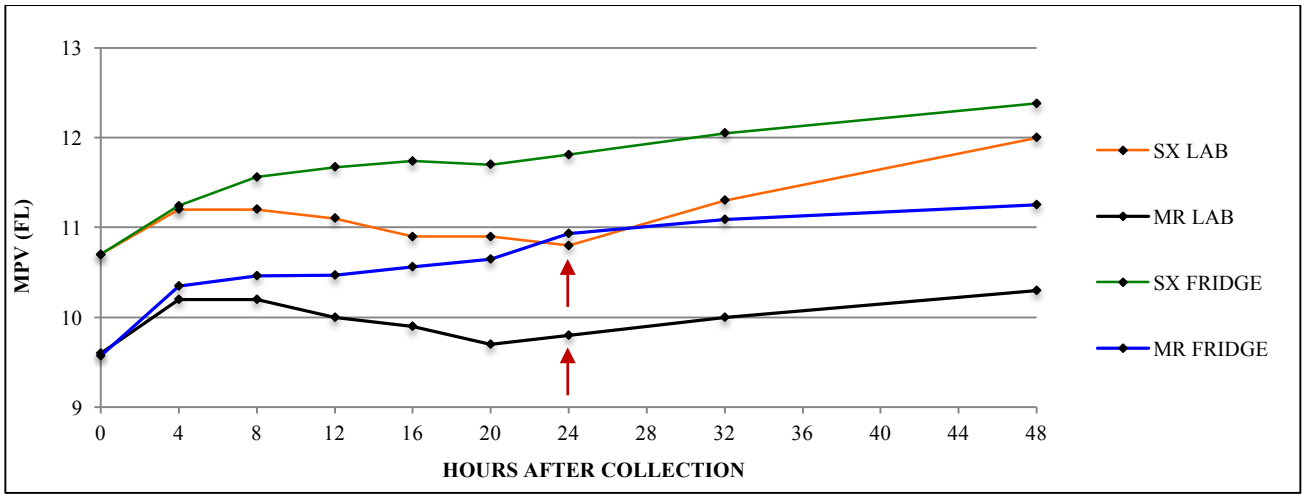


Figure 3. Serial MPV measurements over time in Sysmex (SX) and Mindray (MR) analysers in samples stored at 22-24°C (LAB) and 2-8°C (FRIDGE) temperatures.

The MPV in both analyzers was unstable, with 24 hours of storage at 22-24°C being the latest by which a sample could be analysed to get accurate results on the Sysmex analyser (indicated by red arrows in figure 3). Beyond the stable time periods, there was a steady rise in MPV with time.

Due to the lack of stability of IPF and MPV on Mindray, only Sysmex XN9000 was used for further study purpose.

IV. CHARACTERISATION OF IPF IN PATIENTS WITH THROMBOCYTOPENIA

During the study period, we were able to recruit 200 patients who fulfilled our inclusion criteria. From this, we excluded 4 patients who did not have a final diagnosis because they were lost to follow up. Our working sample size comprised 196 patients.

(A) PATIENT DEMOGRAPHICS

Geographical regions: There was an even distribution of patients coming from all regions of the country. However, the local population accounted for 25% of the total patient number. Almost half (45%) of the patient population in our study were from Bangladesh and eastern states of India like West Bengal, Jharkhand, Bihar, Orissa, Tripura, Meghalaya and Assam.

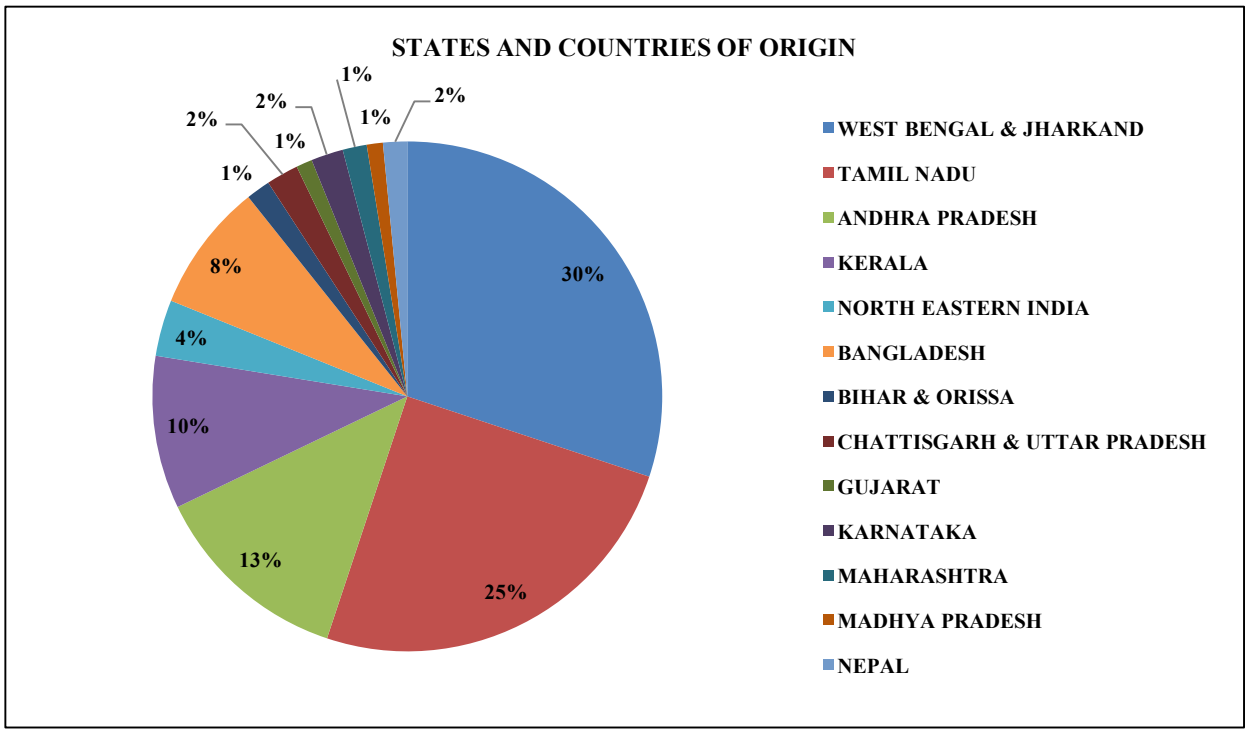


Figure 4. The geographical distribution of patients with thrombocytopenia.

Age: The majority of patients with thrombocytopenia (71%) were adults. The age at presentation ranged from 4 months to 76 years with a mean age of 32 years (Figure 5). All patients who were under the age of 18 were considered as children.

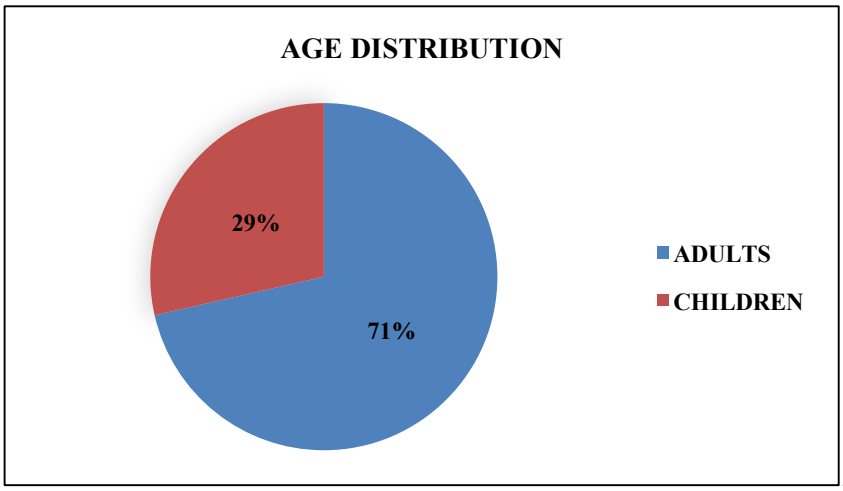


Figure 5. The age wise distribution of patients with thrombocytopenia.

Gender distribution: There was an almost equal distribution of thrombocytopenia among males and females (Figure 6).

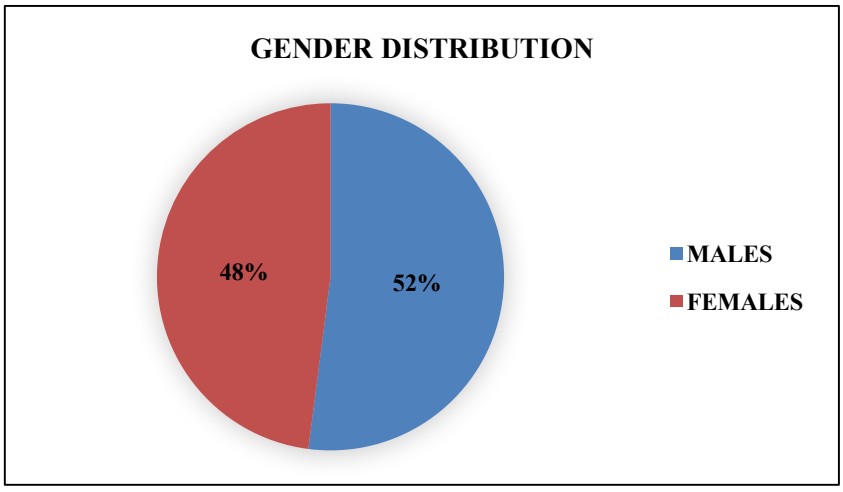


Figure 6. The gender distribution of patients with thrombocytopenia.

(B) ETIOLOGIES OF THROMBOCYTOPENIA:

The most common cause of thrombocytopenia in our study population fell under the broad category of platelet production defects (87 patients; 44%). 67 patients (34%) had thrombocytopenia due to increased peripheral destruction of platelets and 42 patients had other causes of thrombocytopenia which did not fall under any of the 2 main categories (Figure 7).

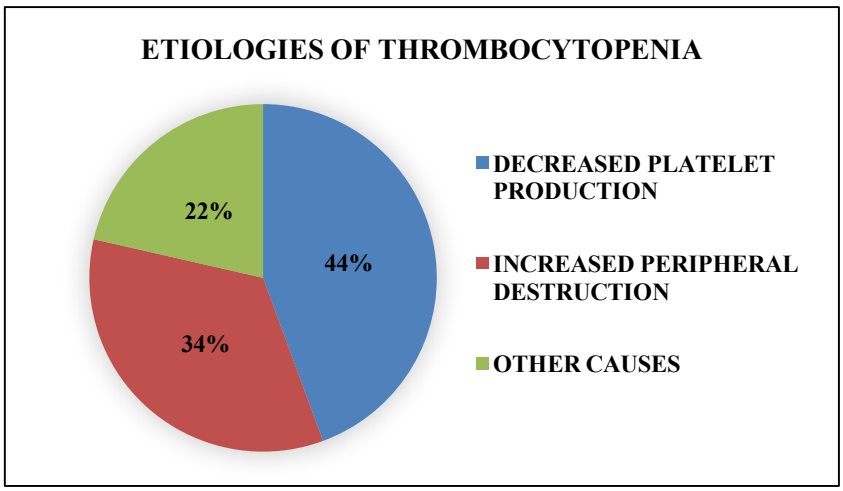


Figure 7. The distribution of etiologies of thrombocytopenia.

The individual patient diagnoses under each broad category of thrombocytopenia were as follows:

Table 5. Causes of peripheral platelet destruction.

ETIOLOGY	NUMBER
Acute and Persistent Immune thrombocytopenic purpura (ITP)	30
Chronic ITP	17
Hypersplenism	20
Total	**67**

The majority of patients (70%) with thrombocytopenia due to a peripheral destruction of platelets had ITP. The remaining patients had hypersplenism due to chronic liver disease or thalassemia (Table 5).

Table 6. Causes of decreased platelet production.

ETIOLOGY	**NUMBER**
Aplastic anaemia	47
Leukaemia	21
Lymphoma/Myeloma	7
Myelodysplastic syndromes (MDS) & Myeloproliferative neoplasms (MPN)	12
Total	**87**

Majority of patients (54%) with a hypoproductive cause of thrombocytopenia were diagnosed to have aplastic anaemia followed by 32% of patients having a marrow infiltrative pathology like leukemia, lymphoma or myeloma (Table 6).

Table 7. Other causes of thrombocytopenia.

ETIOLOGY	NUMBER
Constitutional ethnic macrothrombocytopenia	12
Megaloblastic anaemia	6
Paroxysmal nocturnal haemoglobinuria	4
Infections	3
Chronic kidney disease	3
Bernard Soulier syndrome	2
Wiskott Aldrich syndrome	1
Kasabach Merritt syndrome	1
Drug induced	1
Osteopetrosis	1
MDS or MPN with adequate megakaryocytes	8
Total	**42**

Out of the patients with thrombocytopenia due to causes that did not clearly fit into either of the two broad categories described above, 28% had constitutional ethnic macrothrombocytopenia and 14% had megaloblastic anaemia (Table 7).

(C) PLATELET PARAMETERS IN PATIENTS WITH THROMBOCYTOPENIA:

The median impedance platelet count in all patients with thrombocytopenia was 23500/µl, ranging from a minimum of 1000/µl to a maximum of 97000/µl.

Table 8. Platelet parameters in patients with thrombocytopenia due to decreased production of platelets or increased destruction of platelets and the statistical significance between the two groups.

PARAMETER	DECREASED PRODUCTION **[MEDIAN (IQR)]**	PERIPHERAL DESTRUCTION **[MEDIAN (IQR)]**	P-VALUE
PLT I (/µl)	16000 (9000 - 36000)	30000 (12000 - 56000)	**<0.05**
PLT F (/µl)	15000 (7000 - 29000)	34000 (12000 - 57000)	**<0.05**
Smear platelet count (/µl)	11250 (4500 - 24750)	24750 (12000 - 46500)	**<0.05**
IPF (%)	6.6 (4.6 - 10.1)	15.5 (9.9 - 27.8)	**<0.05**
IPF (%) (Excluding North East India)	5.5 (4.0 - 8.2)	15.2 (9.8 - 23.5)	**<0.05**
PCT (%)	0.03 (0.02 - 0.06)	0.07 (0.05 - 0.09)	**<0.05**
PARAMETER	**[MEAN ± 2SD]**	**[MEAN ± 2SD]**	**P-VALUE**
MPV (fL)	10.4 (4.8 - 16)	9.7 (5.9 - 13.5)	0.38
PDW (fL)	13.4 (9.6 - 17.2)	16.1 (10.1- 22.1)	**<0.05**
P-LCR (%)	36 (17.8 - 54.2)	44.6 (26 - 63.2)	**<0.05**

Since patients from the North Eastern parts of India, Bangladesh and Nepal have a high incidence of ethnic macrothrombocytopenia, we created another subset by excluding all patients from West Bengal, Jharkhand, Orissa, Bihar, Tripura, Meghalaya, Assam, Bangladesh and Nepal and looked at the IPF values in these individuals (N=48). The median IPF dropped from 6.6% (all cases of hypo-production) to 5.5% when the North Eastern patients were excluded.

Platelet count, IPF, PCT, PDW and P-LCR were all significantly lower in patients with thrombocytopenia due to decreased production than in patients with a peripheral destruction of platelets. MPV did not show a significant difference between the two groups (Table 8).

Table 9. Platelet parameters in patients with ethnic macrothrombocytopenia and in patients with increased destruction of platelets

PARAMETER	MACROTHROMBOCYTOPENIA **[MEDIAN (IQR)]**	PERIPHERAL DESTRUCTION **[MEDIAN (IQR)]**	P-VALUE
PLT I (/μl)	47500 (45000 - 56000)	30000 (12000 - 56000)	**<0.05**
PLT F (/μl)	67500 (59500 - 78000)	34000 (12000 - 57000)	**<0.05**
Smear platelet count (/μl)	66000 (59250 - 79500)	24750 (12000 - 46500)	**<0.05**
IPF (%)	47.6 (38.9 - 49.8)	15.5 (9.9 - 27.8)	**<0.05**
PARAMETER	**[MEAN ± 2SD]**	**[MEAN ± 2SD]**	**P-VALUE**
MPV (fL)	10.5 (5.6 – 15.5)	9.7 (5.9 - 13.5)	0.38

Table 10. Platelet parameters in patients with ethnic macrothrombocytopenia and in patients with decreased production of platelets

PARAMETER	MACROTHROMBOCYTOPENIA **[MEDIAN (IQR)]**	DECREASED PRODUCTION **[MEDIAN (IQR)]**	P-VALUE
PLT I (/μl)	47500 (45000 - 56000)	16000 (9000 - 36000)	**<0.05**
PLT F (/μl)	67500 (59500 - 78000)	15000 (7000 - 29000)	**<0.05**
Smear platelet count (/μl)	66000 (59250 - 79500)	11250 (4500 - 24750)	**<0.05**
IPF (%)	47.6 (38.9 - 49.8)	6.6 (4.6 - 10.1)	**<0.05**
PARAMETER	**[MEAN ± 2SD]**	**[MEAN ± 2SD]**	**P-VALUE**
MPV (fL)	10.5 (5.6 – 15.5)	10.4 (4.8 - 16)	1

Patients with ethnic macrothrombocytopenia had significantly higher IPF and platelet counts than patients with either of the other two broad categories of thrombocytopenia (Table 10). PCT, PDW and P-LCR were not analysed as they were not provided by the analyser in patients with giant platelets due to lack of generation of a histogram.

The bone marrow trephine biopsy reports where available were reviewed for all patients. They were classified into two groups: where there appeared to be adequate megakaryocytes in the marrow and those with decreased megakaryocytes and the IPF was compared between the two groups.

Table 11. IPF in patients with adequate bone marrow megakaryocytes and decreased bone marrow megakaryocytes.

	ADEQUATE MEGAKARYOCYTES	**DECREASED MEGAKARYOCYTES**	**P-VALUE**
Number of patients	54	33	
IPF (Median [IQR])	14.1 (8.1-25.3)	8.3 (5.4-12.8)	<0.05

Median IPF in patients with adequate bone marrow megakaryocytes was 14.1% which was significantly higher than in patients with decreased bone marrow megakaryocytes who had a median IPF of 8.3% (Table 11).

(D) UTILITY OF IPF TO DISTINGUISH BETWEEN PATIENTS WITH THROMBOCYTOPENIA DUE TO PERIPHERAL DESTRUCTION AND DECREASED PRODUCTION OF PLATELETS:

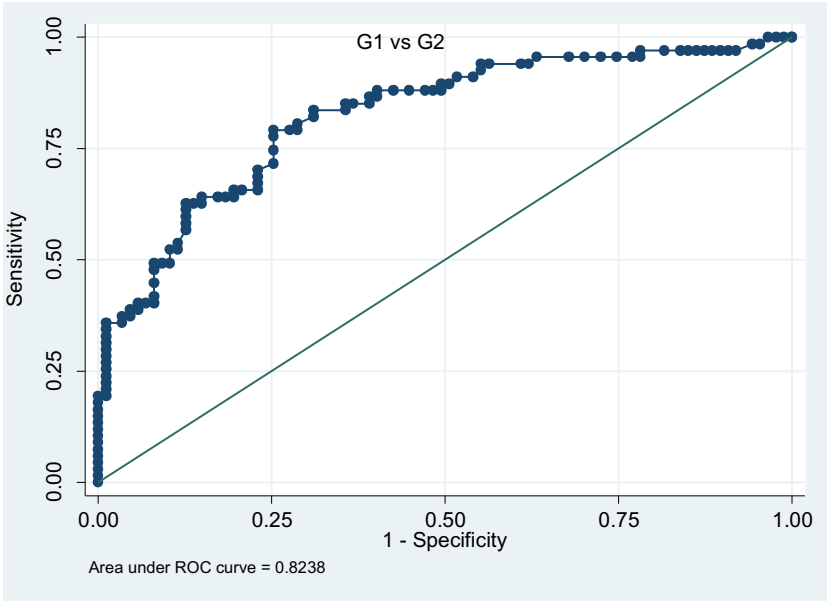


Figure 8. ROC curve – IPF in patients with thrombocytopenia due to decreased production and peripheral destruction of platelets.

An IPF of ≥ 9.3% can distinguish thrombocytopenia due to peripheral destruction of platelets from hypoproductive thrombocytopenia with a sensitivity of 80% and a specificity of 71% (Figure 8).

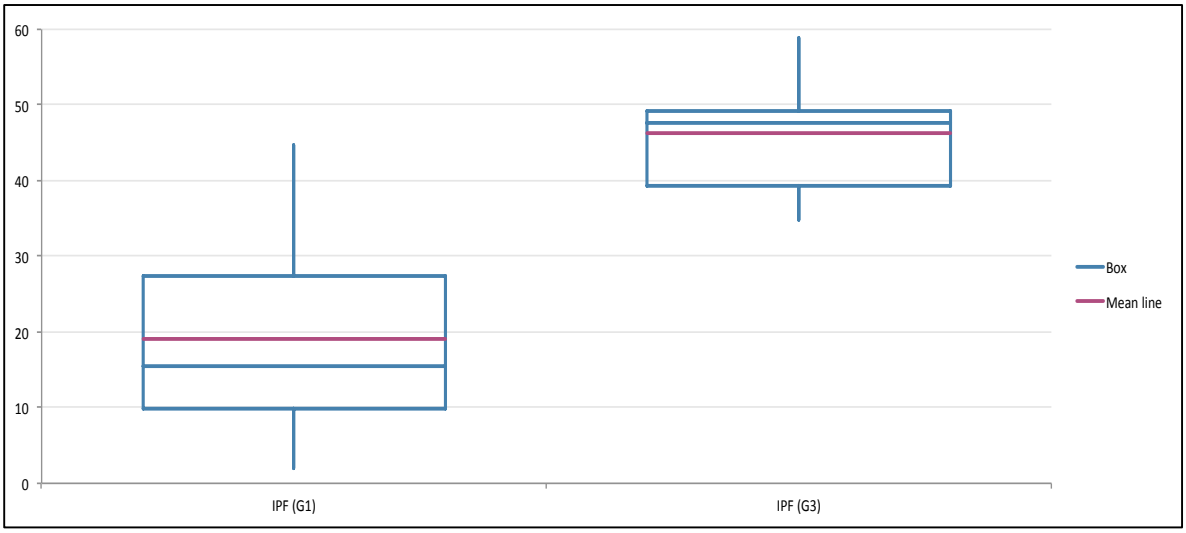


Figure 9. Box plot highlighting the clear separation between the mean IPF values in patients with ethnic macrothrombocytopenia (G3) and in patients with peripheral platelet destruction (G1).

Table 12. Minimum and maximum IPF values in the two patient groups from which the box plot was derived.

Patient group	Mean	Minimum	Maximum
Peripheral destruction	18.9	1.9	44.8
Macrothrombocytopenia	46.2	34.8	58.9

An IPF of ≥ 45% can distinguish between thrombocytopenia due to peripheral destruction of platelets from ethnic macrothrombocytopenia with 100% specificity and 66% sensitivity (Figure 9 and Table 12).

(E) PLATELET MORPHOLOGY IN THROMBOCYTOPENIA.

Slides were prepared for all patients with thrombocytopenia and stained with modified Wright Giemsa stain (annexure for SOP) using the Beckman Coulter Automated Slidemaker and Stainer. These were reviewed and the platelets were categorized into three groups as shown in the table below (Table 13).

Table 13. Morphological variation of platelets (size) in patients with thrombocytopenia due to decreased production and increased destruction of platelets (Median percentage and ranges).

ETIOLOGY	N	SMALL PLATELETS	LARGE PLATELETS	GIANT PLATELETS
DECREASED PRODUCTION	81	78% (30-100%)	20% (0-70%)	2% (0-20%)
PERIPHERAL DESTRUCTION	67	70% (10-100%)	28% (0-82%)	2% (0-20%)
ETHNIC	12	41% (24-100%)	57% (0-72%)	2% (0-8%)

Patients with peripheral destruction of platelets had a slightly higher percentage of larger platelets. Patients with ethnic macrothrombocytopenia seemed to have had a higher number of large platelets as compared to the other two groups.

Table 14. Colour of platelet cytoplasm in Wright Giemsa stained slides.

ETIOLOGY	N	GREY	GREY BLUE	BLUE	NOT ASSESSED
DECREASED PRODUCTION	87	21	58	2	6
INCREASED DESTRUCTION	67	8	55	3	1
ETHNIC	12	1	9	2	0

Table 14 shows that more number of patients with peripheral destructive or ethnic macrothrombocytopenia had a higher number of platelets with a more bluish tinge to the cytoplasm. However, this finding was not statistically significant (p value = 0.06).

V. TRENDS OF IPF IN PATIENTS POST HSCT.

During the study period, there were 24 patients with aplastic anaemia who underwent HSCT out of which 4 patients expired before achieving platelet engraftment. One patient did not have serial IPF measurements till date of engraftment. These 5 patients were excluded from the study. 2 patients were undergoing a second transplant after previous secondary graft failure. 18 patients with Beta Thalassemia major underwent HSCT during the study period but 7 patients were excluded as they did not have serial IPF measurements till date of engraftment. 7 patients with lymphoma underwent autologous HSCT during the study period out of which 3 patients were excluded (one patient expired before platelet engraftment and 2 patients did not have serial IPF measurements till date of engraftment). Out of the 12 patients who underwent autologous HSCT for Multiple myeloma, 3 patients were excluded as they did not have serial IPF measurements till date of engraftment.

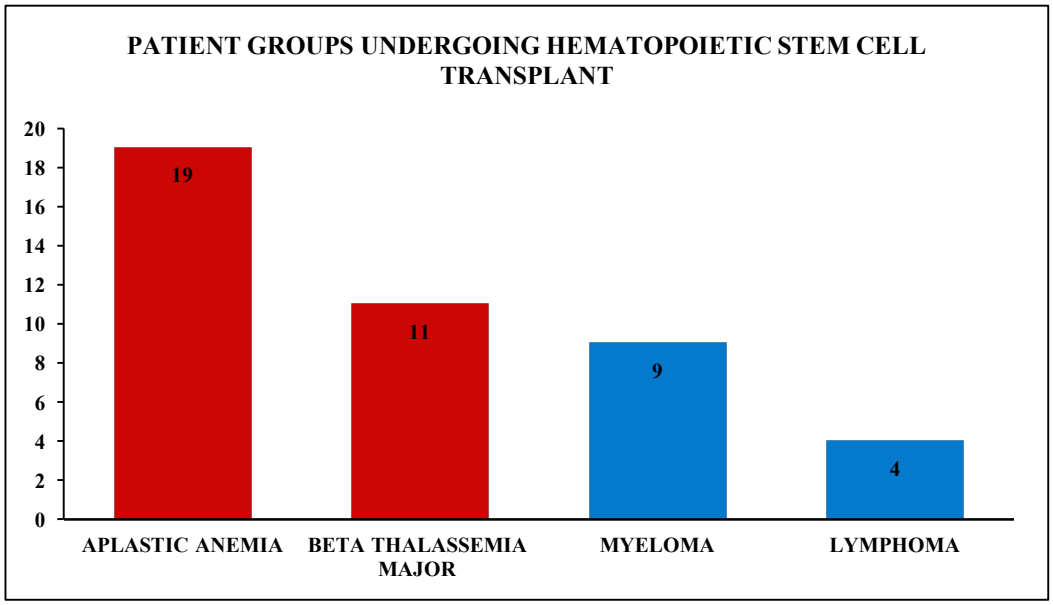


Figure 10. Total number of patients in different disease groups who underwent HSCT.

(A) PATIENT DEMOGRAPHICS, HLA CROSS MATCH STATUS AND CONDITIONING REGIMENS USED:

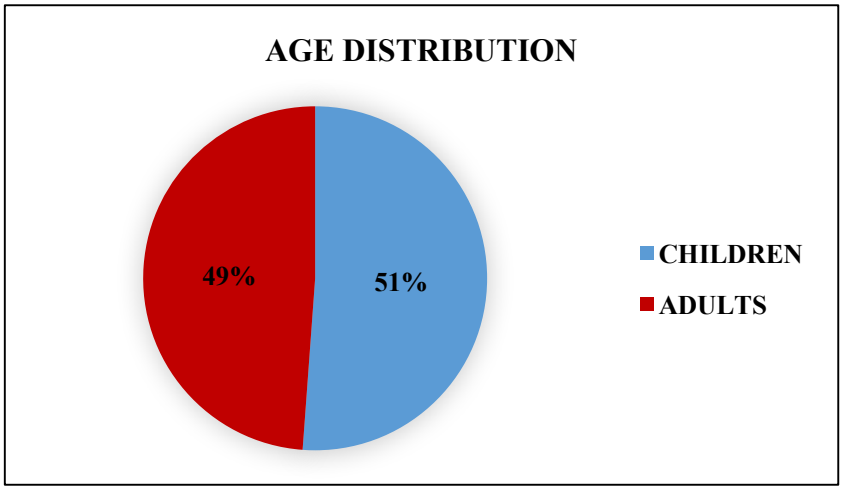


Figure 11. Age distribution of patients undergoing HSCT.

There was an almost equal number of children and adult patients in our study groups (Figure 11). The ages ranged from 2 years to 59 years.

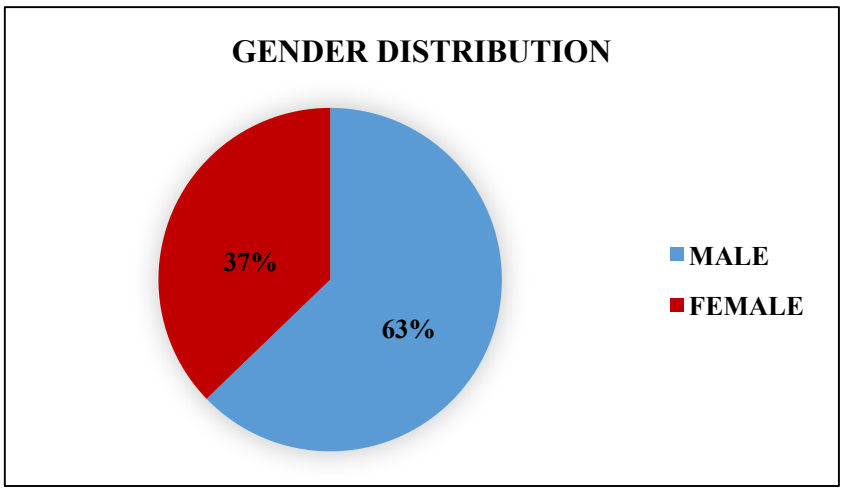


Figure 12. Gender distribution of patients undergoing HSCT.

63% (27) of the patients were male and 16 patients were female (Figure 12).

Table 15. HLA (Human leukocyte antigen) match status of allogenic HSCT patients.

HLA CROSS MATCH STATUS	NUMBER OF PATIENTS
Haplomatched	3
Fully matched sibling donor	21
Fully matched family donor	3
Fully matched unrelated donor	1
2 antigen mismatched sibling donor	1
2 antigen mismatched unrelated donor	1
Total	**30**

21 out of 30 patients had a fully HLA cross matched sibling donor (Table 15).

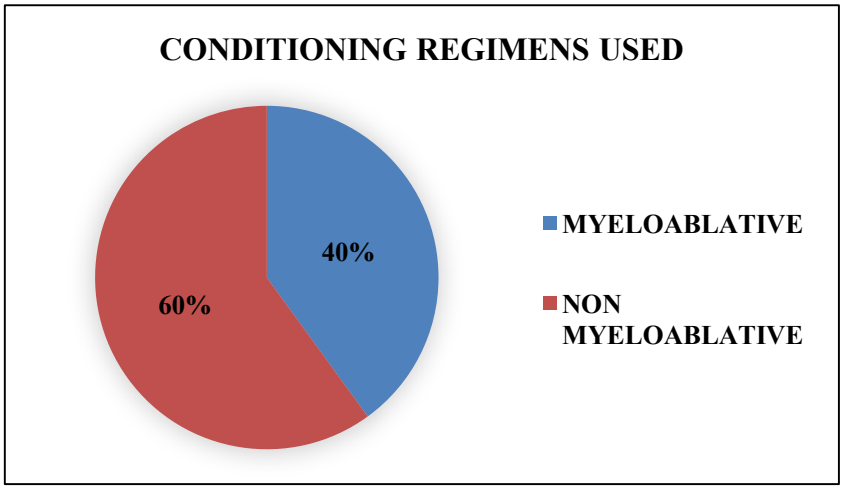


Figure 13. Conditioning regimens used in patients undergoing allogenic HSCT.

60% of patients undergoing allogenic transplant had a non-myeloablative conditioning regimen (Figure 13). All patients with myeloma and lymphoma who underwent autologous HSCT were conditioned with Melphalan and BEAM protocol (Carmustine, Etoposide, Cytarabine and Melphalan) respectively.

(B) TRENDS OF IPF IN PATIENTS WITH APLASTIC ANAEMIA POST ALLOGENIC HSCT:

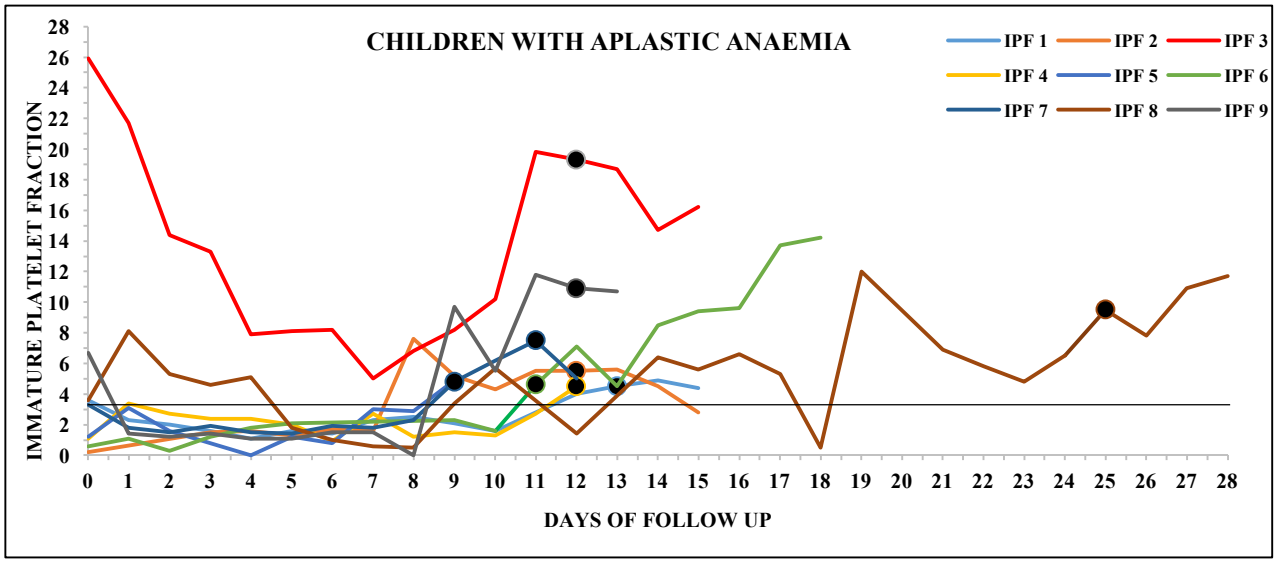


Figure 14. Serial measurements of IPF in children with Aplastic anaemia post allogenic HSCT. Black circles indicate day of platelet recovery. Black line indicates upper limit of age specific reference interval for IPF (3.1%).

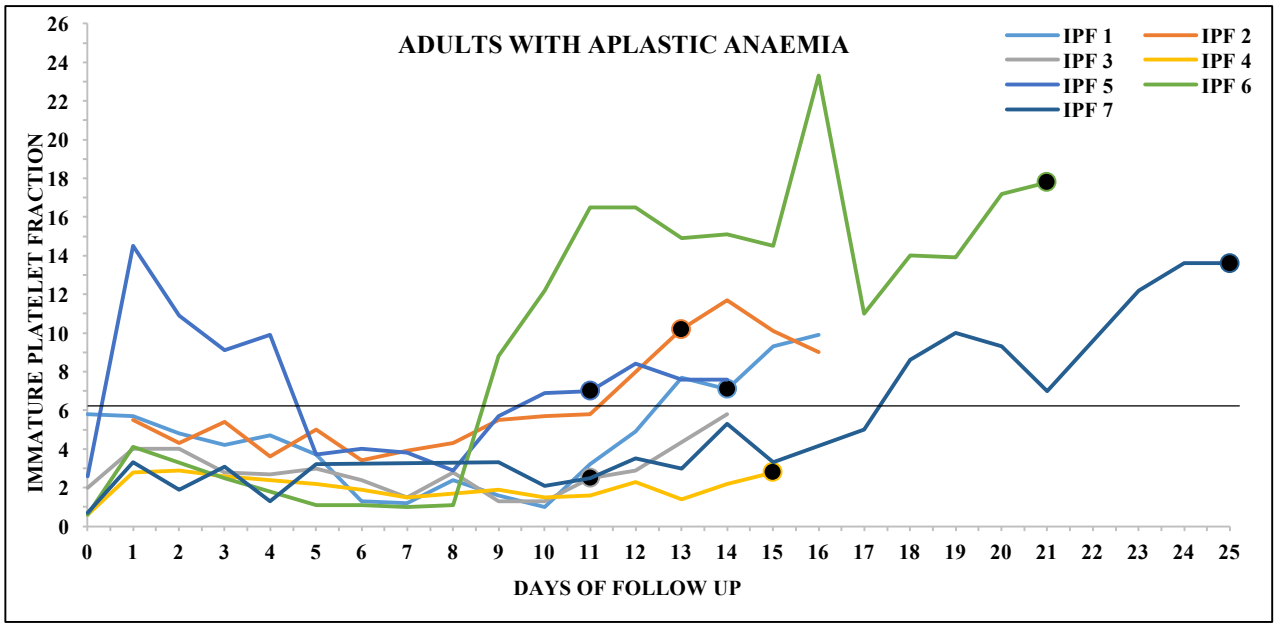


Figure 15. Serial measurements of Immature platelet fraction (%) in adults with Aplastic anaemia post allogenic transplant. Black circles indicate day of platelet recovery. Black line indicates upper limit of age specific reference interval for IPF (6.1%).

IPF in patients with Aplastic Anaemia (Post Allogenic BMT): In this group of patients, serial measurements of IPF from Day 0 of transplant (i.e. day of infusion of peripheral blood stem cells) up until the day of platelet recovery and beyond have been represented in figures 14 and 15. On applying the upper limit of our reference interval for IPF as a cut off, 5 out of 7 adults and 8 out of 9 children went above the upper limit of reference intervals for the respective groups a median of 1 day and 1.5 days before platelet recovery respectively. In total, this showed that in 81% of patients, IPF showed an early indication of impending increase in platelet count.

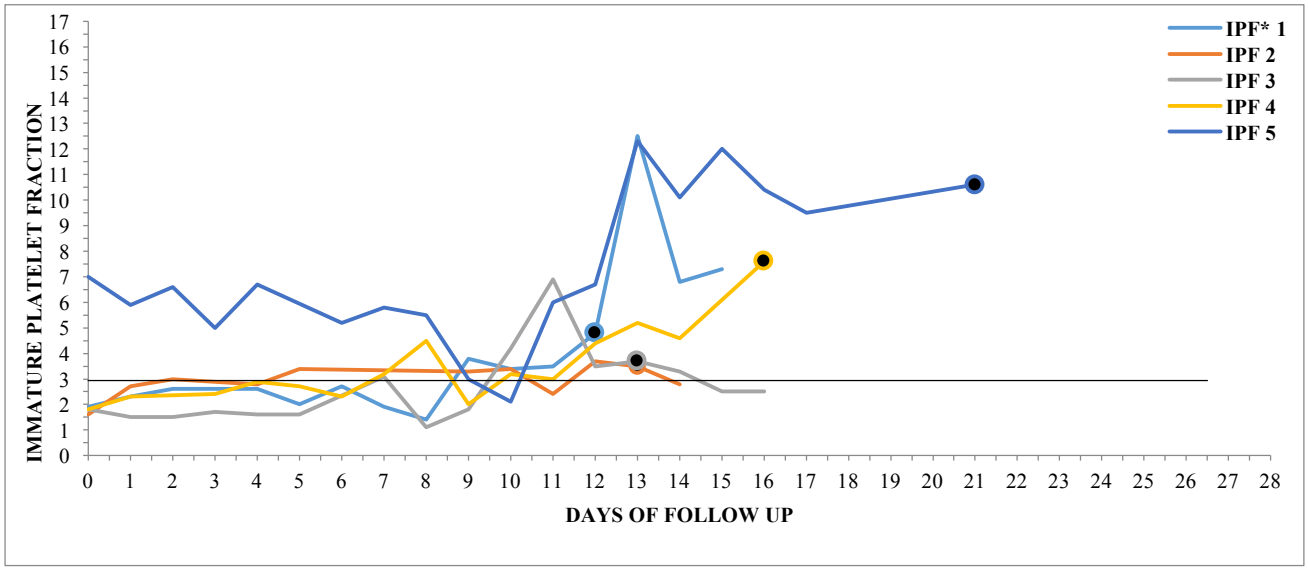


Figure 16. Serial measurements of Immature platelet fraction (%) in children with Thalassemia post allogenic transplant (patients 1 - 5). Black circles indicate day of platelet recovery. Black line indicates upper limit of normal reference range for IPF in children (3.1 %).

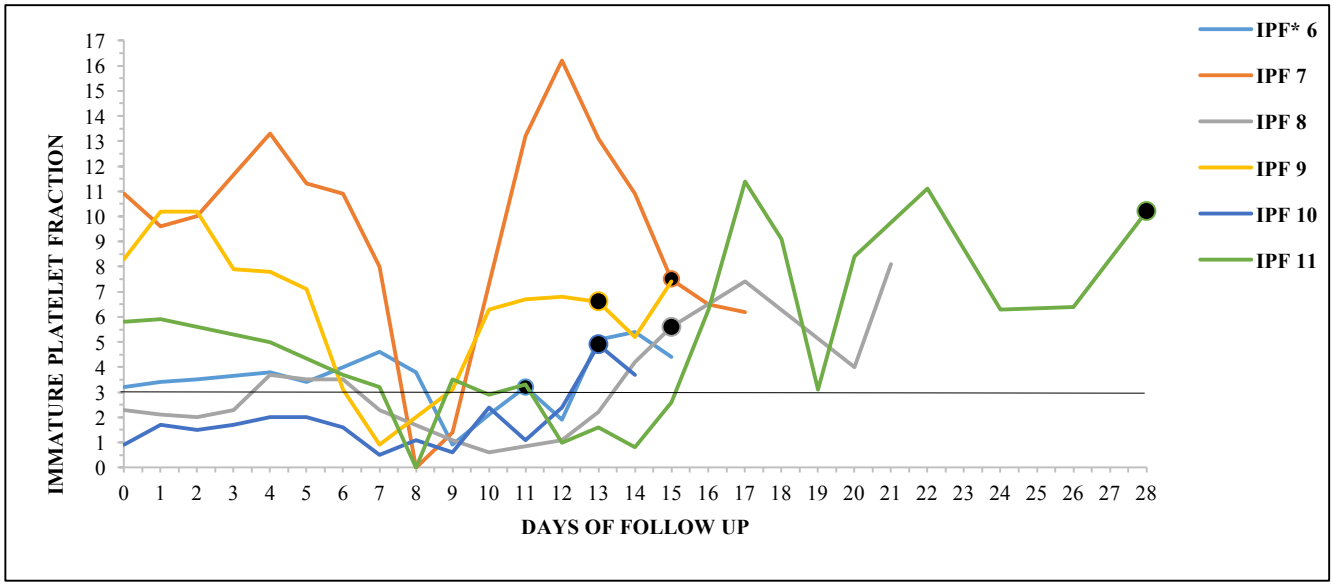
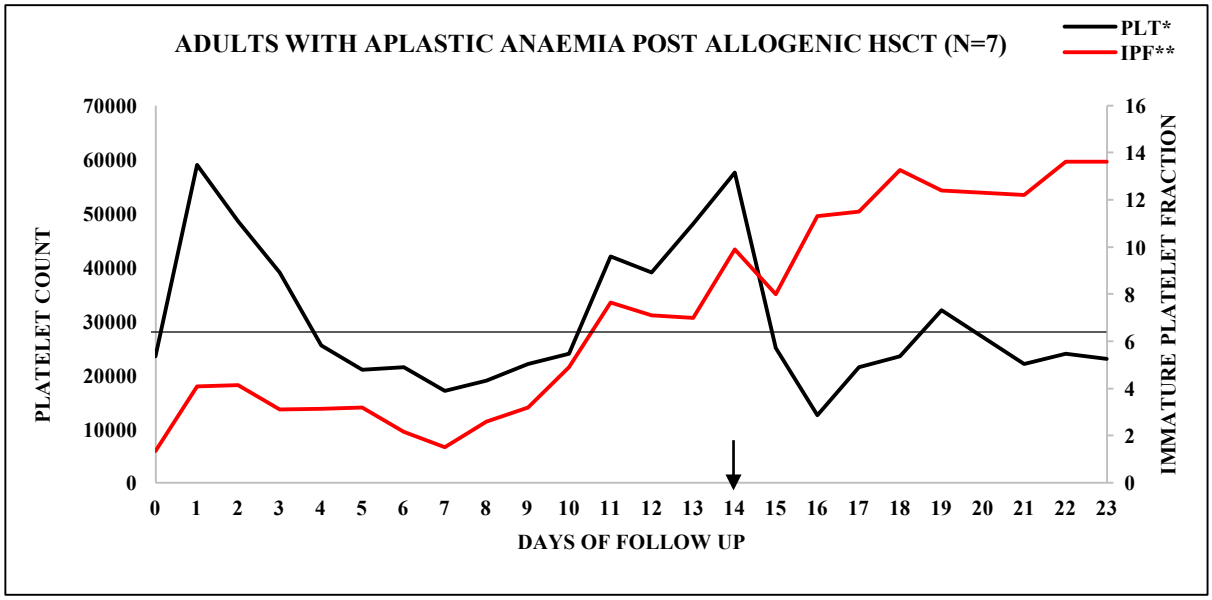


Figure 17. Serial measurements of Immature platelet fraction (%) in children with Thalassemia post allogenic transplant (patients 6 - 11). Black circles indicate day of platelet recovery. Black circles indicate day of platelet recovery. Black line indicates upper limit of normal reference range for IPF in children (3.1 %).

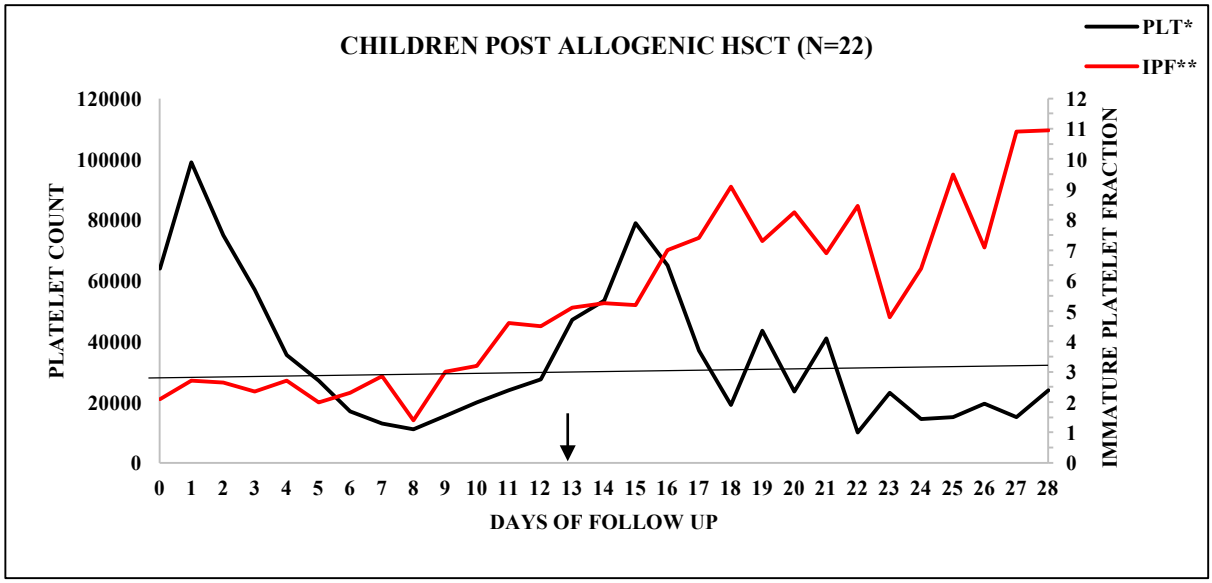
Figures 16 and 17 represent serial measurements of IPF from Day 0 of transplant (i.e. day of infusion of peripheral blood stem cells) up until the day of platelet recovery and beyond in patients with Thalassemia. By using the upper limit of our reference range for IPF in children as a cut off, i.e. 3.1%, we observed that the IPF values of all patients went above the reference range before the day of platelet recovery (median of 3.5 days before recovery).



* PLT – Median platelet count. ** IPF – Median immature platelet fraction.

Figure 18. Trends of Median IPF and Median platelet count over time in all adult patients post allogenic HSCT who engrafted (N=7). Black arrow indicates the median day of platelet recovery. Black line indicates age specific reference interval for IPF.

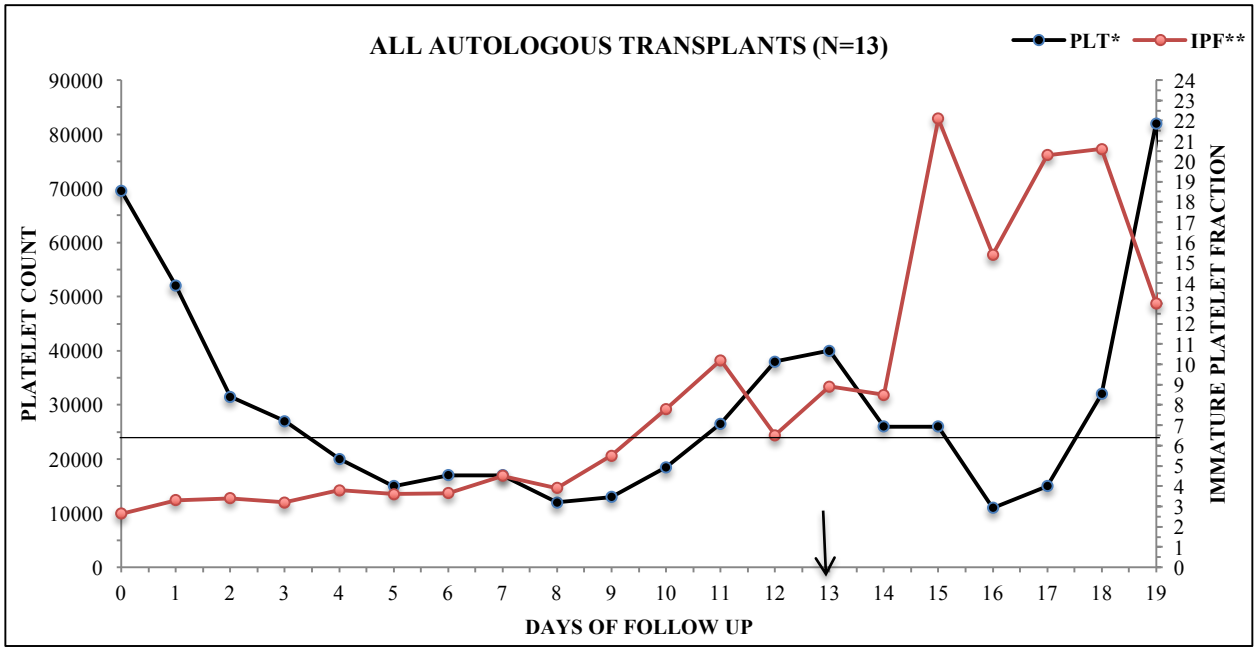
Figure 18 shows that the IPF went above the age specific reference interval around 3 days before the median day of platelet recovery. Beyond day 15, the graph shows a drop in the platelet count due to 2 patients who engrafted only after Day + 20 post-transplant.



* PLT – Median platelet count. ** IPF – Median immature platelet fraction.

Figure 19. Trends of Median IPF and Median platelet count over time in all children who engrafted post allogenic HSCT (N=22). Black arrow indicates the median day of platelet recovery. Black line indicates age specific reference interval for IPF.

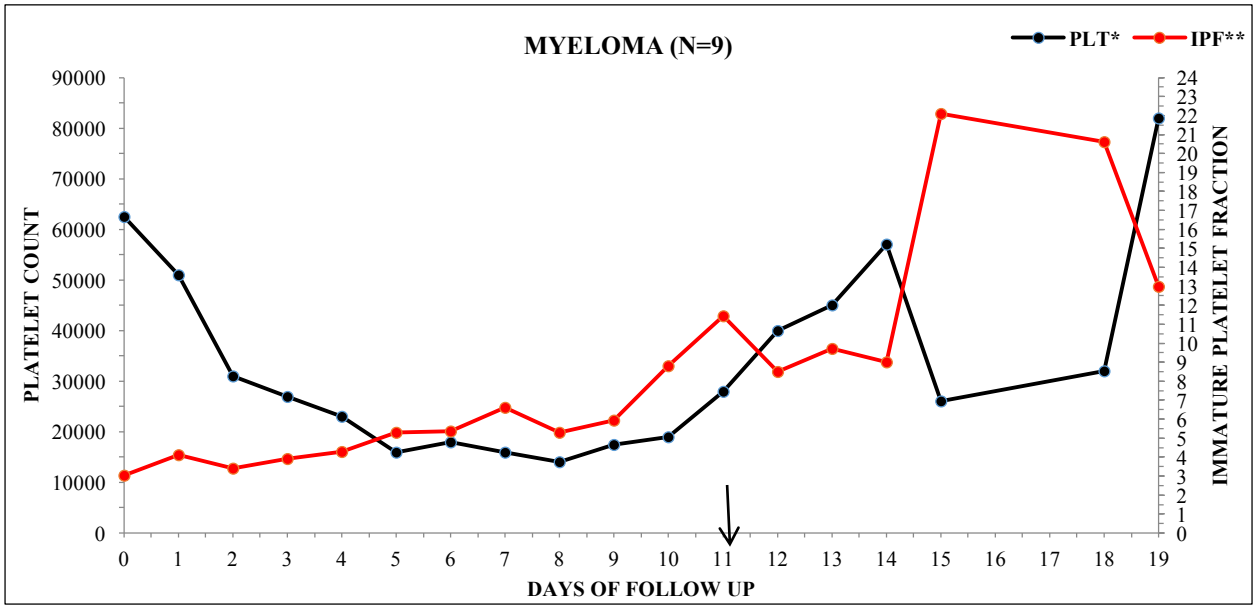
Figure 19 shows that the IPF went above the age specific reference interval around 3 days before the median day of platelet recovery. Beyond day 15, the graph shows a drop in the platelet count due to 3 patients who engrafted only after Day + 20 post-transplant.



* PLT – Median platelet count. ** IPF – Median immature platelet fraction.

Figure 20. Trends of Median IPF and Median platelet count over time in all patients post autologous HSCT (N=13). Black arrow indicates the median day of platelet recovery. Black line indicates age specific reference interval for IPF.

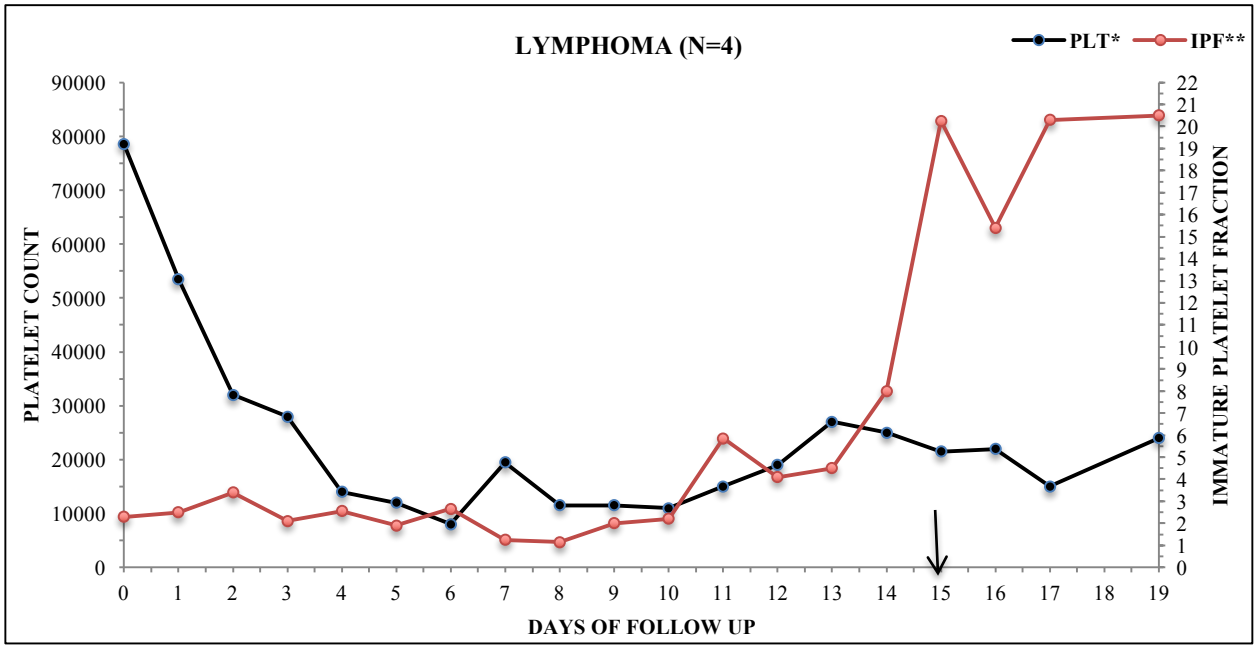
Figure 20 shows that the IPF went above the age specific reference interval around 3 days before the median day of platelet recovery. 9 out of the 13 patients showed an increase in IPF above the upper limit of reference interval (>6.1%) a median of 3 days before the day of platelet recovery.



* PLT – Median platelet count. ** IPF – Median immature platelet fraction

Figure 21.Trends of Median IPF and Median platelet count over time in patients with Multiple myeloma post autologous HSCT (N=9). Black arrow indicates the median day of platelet recovery.

Figure 21 shows that the IPF started to show an increasing trend around 3 days before the day of platelet recovery. 7 out of the 9 patients showed an increase in IPF above the upper limit of reference interval (>6.1%) before the day of platelet recovery.



* PLT – Median platelet count. ** IPF – Median immature platelet fraction.

Figure 22. Trends of Median IPF and Median platelet count over time in patients with Lymphoma post autologous HSCT (N=4). Black arrow indicates the median day of platelet recovery.

Figure 22 shows that the IPF started to show an increasing trend around 3 days before the day of platelet recovery. 2 out of the 4 patients showed an increase in IPF above the upper limit of reference interval (>6.1%) before the day of platelet recovery.

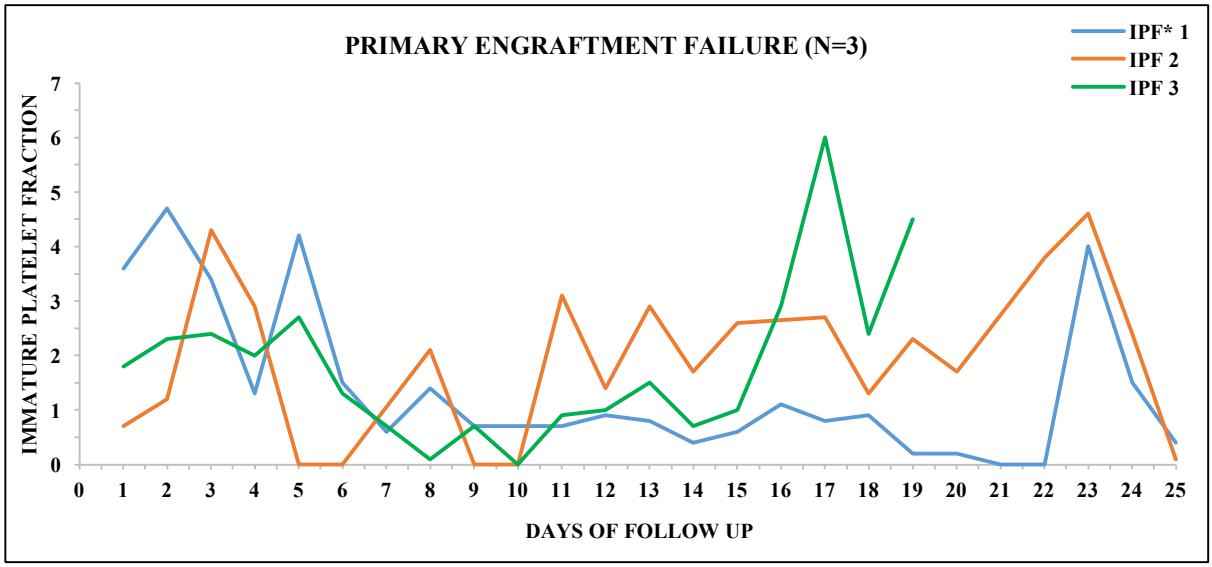


Figure 23. Serial IPF measurements of 3 patients who underwent allogenic HSCT for aplastic anaemia and had primary engraftment failure.

Figure 23 shows that there was a marked variation in IPF with no sustained rise seen in serial IPF values.

DISCUSSION

REFERENCE INTERVALS FOR PLATELET PARAMETERS

Age specific reference intervals for the following parameters: MPV, IPF, PDW, P-LCR and PCT from 248 healthy adults and 27 healthy children on both Sysmex XN 9000 and Mindray BC 6800 platforms were determined. The data was tested for normality of distribution. Since none of these parameters had a normal distribution we used 5^{th} and 95^{th} percentiles as reference ranges. This lack of a normal distribution for platelet parameters has been reported previously and hence authors have used different methods for reference range calculation like Mean ± 2SD, 25^{th} and 75^{th} percentiles or minimum and maximum (98,99). Our reference interval of IPF in adults was 1.1 – 6.1% and 1.6 – 8.9% in the Sysmex and Mindray analysers respectively. The values we obtained for the Sysmex analyser were in keeping with the reference ranges obtained in studies by Briggs *et al*. and Seo *et al*. (59,100) but differ from those quoted in other studies like the one by Jung *et al*. which gives a lower cut-off of 3.2% as the upper limit of normal (99). This difference could be due to differences in the statistical tools used to derive the reference ranges.

Our reference interval for IPF in children was much narrower and lower than that for adults and was taken as 0.4 – 3.1%. Seo *et al*. had reported a similar finding of an IPF nadir occurring between the ages of 2-6 years followed by increasing IPF to reach adult levels beyond 18 years of age (100). This phenomenon could be due to difference in platelet dynamics in children or could also be due to the smaller sample size that we had for the reference range calculation in children.

It has been observed that individuals from North Eastern states of India, Bangladesh and Nepal have a high incidence of a phenomenon called constitutional ethnic

macrothrombocytopenia which is an asymptomatic condition wherein, the individual has giant platelets in the peripheral blood and thrombocytopenia (101). It has also been documented that these individuals have a high IPF (Median 25.5%) (102). Since our institution has a significant proportion of its patient population coming from these states, we calculated reference ranges separately for IPF from 131 healthy individuals from North Eastern India. We obtained a median IPF of 10.4% and a reference interval of 2.1 – 34.8% (5^{th} and 95^{th} percentile).

AGREEMENT BETWEEN THE TWO ANALYSERS

Bland Altman agreement statistics performed between the two analysers showed that majority of the data points fell within the 95% confidence intervals. The Intra-class correlation coefficient was found to be 0.92 (95% CI: 0.81-0.97) which suggested that there was a good agreement between the two analysers. There have been no previous studies that have looked at the agreement between these two analysers.

STABILITY OF PLATELET PARAMENTERS ON BOTH ANALYSERS

Previous literature so far has given contradicting evidence on stability of platelet parameters on Sysmex analysers. Studies by Briggs *et al.* on Sysmex XE series had shown that IPF was stable up to 48 hours of storage (59). However, certain other studies have quoted much lesser stability times varying between 3 – 24 hours with decreasing stability on storage at refrigerated conditions (62,63,83). The Sysmex XN analyser has been reported to have better stability of up to 48 hours with storage at room temperature or refrigerated conditions (27,80). The stability of these parameters

on Mindray BC6800 has not been studied so far. In our study, we found that IPF on Sysmex XN was stable up to 48 hours when stored at either room or refrigerated temperatures which was in keeping with previous studies. However, IPF on Mindray was stable only up to 8 hours on storage at room temperature and up to 4 hours on storage in refrigerated conditions beyond which there was a statistically significant rising trend in IPF values. MPV was equally unstable in both analysers with increasing values being obtained beyond 4 hours of storage at refrigerated temperatures. Platelet counts were stable up to 48 hours in both analysers. This lack of stability in IPF on Mindray could be due to the different fluorescent dye and effect of analytic reagents and methods in the chamber that is used in this analyser.

CHARACTERISATION OF IPF IN THROMBOCYTOPENIA

One of the objectives of the study was to study IPF in different patient groups, primarily to see if this parameter was different in patients who had a peripheral platelet destruction over those that had a bone marrow production problem. We also hypothesized that the trends of IPF during bone marrow recovery in patients who had undergone stem cell transplant for various hematological conditions could help to predict those who would display platelet recovery.

Patient demographics: There was an even distribution of patients coming from all parts of the country. However, the local population accounted for only 25% of the total patient number. 45% of our patient population was from Bangladesh, Nepal and

eastern states of India like West Bengal, Jharkhand, Bihar, Orissa, Tripura, Meghalaya and Assam.

71% of patients were adults with an age range of 4 months to 72 years. Mean age at presentation with thrombocytopenia was 32 years.

There was an almost equal distribution of thrombocytopenia among men (52%) and women (48%), showing a slight male predominance.

Etiologies of thrombocytopenia: The most common cause of thrombocytopenia in patients presenting to our institution during the study period was a decreased production of platelets from the marrow i.e. a hypoproductive cause of thrombocytopenia (44%, 87 out of 198 patients). This hypoproduction of platelets was due to a variety of conditions, the most common of which was aplastic anaemia (54%, 47 patients) and marrow infiltrative pathologies like leukemia, lymphoma and myeloma (32%, 28 patients). The remaining 14% of patients had Myelodysplastic syndrome or Myeloproliferative neoplasms.

Thrombocytopenia due to a peripheral destruction of platelets was seen in 34% of patients. Of this, immune thrombocytopenic purpura (ITP) was the most common cause (70%, 30 out of 67 patients). In the remaining 30% of patients, the peripheral platelet destruction was due to sequestration in an enlarged spleen. This finding is in agreement with the global data that ITP is the commonest cause of isolated thrombocytopenia (11).

About a quarter of patients (42 out of 198 patients, 22%) had thrombocytopenia due to causes which did not clearly fit into an isolated problem of platelet production or platelet destruction. Of these, the common causes were constitutional ethnic macrothrombocytopenia (28%) and megaloblastic anaemia (14%). Other causes encountered included Paroxysmal nocturnal hemoglobinuria, Wiskott-Aldrich syndrome, chronic kidney disease, infections, MDS and MPN (with adequate bone marrow megakaryocytes), Bernard Soulier syndrome, Osteopetrosis and drug induced. We could not find any data on the common causes of thrombocytopenia with or without other cytopenias in the Indian population or others, in literature.

PLATELET PARAMETERS IN PATIENTS WITH THROMBOCYTOPENIA

Hypoproductive thrombocytopenia: Median impedance platelet counts in patients with a hypoproductive cause of thrombocytopenia was 16000/µl (IQR: 9000/µl – 36000/µl). Median fluorescent platelet count (PLT-F) and manual smear platelet count was also similar being 15000/µl and 11250/µl respectively. The median IPF in this group was 6.6% (IQR: 4.6% – 10.1%) . When we excluded the 39 patients coming from North East India from this group, the median IPF dropped to 5.5% (IQR: 4% - 8.2%). This drop in IPF was probably due to the high incidence of ethnic macrothrombocytopenia in the excluded group of patients which would have caused higher IPF values in them despite their hypoproductive bone marrow state. The median IPF values in this group was significantly higher than the median IPF of our normal control population (Median: 2.5%). This is probably due to the marrow trying to compensate for the thrombocytopenia by producing platelets at a rate which is

higher than that seen in normal individuals in whom there is no stimulus for platelet production in the resting state. Patients with thrombocytopenia due to decreased platelet production (ex: aplastic anaemia, leukaemia, lymphoma involving marrow etc.) have lesser number of megakaryocytes within the marrow (103). So it is possible that even if these remaining megakaryocytes produced platelets at an accelerated rate to compensate for the thrombocytopenia, it will not match the rate of platelet production from a marrow with adequate megakaryocytes which is being stimulated to produce platelets due to a peripheral platelet consumption. Kickler *et al.* (68) had similar values of IPF in hypoproductive thrombocytopenia (Mean: 4.1%, Range: 0.8% - 8.8%) with the mean IPF being higher in patients with hypoproductive thrombocytopenia when compared to normal control population. Abe *et al.* also obtained similar values of IPF in patients with aplastic anaemia (Mean: 6.4%, Range: 2.8% - 10%) (66). Briggs *et al.* and Adly *et al.* also reported that patients with hypoproductive thrombocytopenia have significantly higher IPF values than the control population (59,67).

The mean MPV, PDW and P-LCR in this group was 10.4 fL (± 2 SD: 4.8 – 16 fL), 13.4fL (± 2 SD: 9.6 – 17.2 fL) and 36% (± 2 SD: 17.8 – 54.2%) respectively. The median PCT was 0.03% (IQR: 0.02 – 0.06%). These values differed slightly from that obtained Kaito *et al.* who had reported mean values of MPV, PDW and P-LCR in patients with aplastic anaemia as 10.2 fL, 11.6 fL and 25.7% respectively and from that obtained by Adley *et al.* who had mean values of 10.5 fL, 9.7 fL and 27.6% in patients with haematological malignancies. These differences were probably due to difference in analyser used (Sysmex XE series in the quoted studies) and differences

in the patients groups with hypoproductive thrombocytopenia studied. The median values for PCT in these patients were significantly lower than the normal controls. The MPV and PDW values were also lower though not significantly so. On the other hand, they had a significantly higher P-LCR than the control population.

Thrombocytopenia due to peripheral destruction of platelets: Median impedance platelet counts in patients with a peripheral destructive cause of thrombocytopenia was 30000/µl (IQR: 12000/µl – 56000/µl). Median PLT-F count and smear platelet count was also similar being 34000/µl and 24750/µl respectively. We also found that PLT-F count was similar to PLT-I count when the platelet histograms were normal. When the histogram was abnormal (reflecting the presence of large platelets, numerous small platelets or significant platelet anisocytosis) the PLT-F and PLT-I counts started to differ from each other. The median IPF in this group was 15.5% (IQR: 9.9% – 27.8%) with a range of 1.9 - 44.8% . Kickler *et al.* obtained a mean IPF of 12.8% (Range: 2.3 – 35.4%), Briggs *et al.* reported a mean IPF of 16.8% (Range: 2.3 – 52.1%), Adly *et al.* had a median IPF of 11.8% (Range: 2.8 – 39.8%) and Abe *et al.* reported a mean IPF of 17.4% (Range: 1.2 – 53.2%) (59,66–68). Even though the mean / median IPF and the maximum possible IPF that was recorded was not exactly the same, they were all universally above the upper limit of the normal reference ranges for IPF as was the case in our study as well. The median IPF in this group (15.5%) was also significantly higher than that of the control population (2.5%). The mean MPV, PDW and P-LCR in this group was 9.7 fL (± 2 SD: 5.9 – 13.5 fL), 16.1 fL (± 2 SD: 10.1 – 22.1 fL) and 44.6% (± 2 SD: 26 – 63.2%) respectively. The

median PCT was 0.03% (IQR: 0.02 – 0.06%). Kaito *et al.* and Adly *et al.* reported higher mean values of MPV of 12.2 fL and 11.6 fL respectively in patients with ITP. However, mean values of PDW and P-LCR obtained by Kaito *et al.* were similar to the values that we obtained i.e. 16.8 fL and 42.2% respectively (67,97). Adly *et al.* reported lower mean values of PDW and P-LCR i.e. 14.5 fL and 42.8% respectively and a higher mean value for PCT (0.1%) when compared to our study (67). These minor differences in values could be due to difference in analyser used (Sysmex XE series in the quoted studies) and the fact that all the quoted studies included only patients with ITP in their study groups whereas our study had included patients with hypersplenism as well.

In our study, the mean MPV (9.7fL) and PCT (0.03%) in this patient group was significantly lower than the median MPV (10.7fL) and PCT (0.05%) values of the normal population. However, the mean PDW and P-LCR were significantly higher than that of the control population. These findings suggest that patients with thrombocytopenia due to a peripheral destruction of platelets have more larger sized platelets in circulation and a greater degree of platelet anisocytosis than normal individuals.

Constitutional ethnic macrothrombocytopenia: Median impedance platelet counts in patients with ethnic thrombocytopenia was 47500/µl (IQR: 45000/µl – 56000/µl). Median PLT-F count and smear platelet count was higher being 67500/µl and 66000/µl respectively, which was statistically significant. This difference was probably due to PLT-I count missing out on the large platelets due to their

misclassification as red blood cells by the analyser. The median IPF in this group was 47.6% (IQR: 38.9% – 49.8%) which was significantly higher than the normal control population. The mean MPV was 10.5 fL (2SD: 5.6 - 15.5 fL) which did not differ significantly from that of the normal controls. PDW, PCT and P-LCR were not provided by the analyser because of abnormal platelet histograms in these patients due to the presence of giant platelets. Bhat *et al.* reported a lower median IPF of 25.5% (Range: 8 - 47.1%) in 77 patients with ethnic macrothrombocytopenia from West Bengal. This difference was probably because the population they included in their study also included patients with less severe thrombocytopenia (>100000/μl and < 150000/μl) as well. When they categorised their patients based on different platelet count cut offs, they obtained higher IPF values (30% - 35%) for platelet counts <100000/μl which is more similar to the value obtained by us. They also reported that Sysmex XN analyser failed to give an MPV, PDW, PCT and P-LCR in majority of these cases due to an abnormal platelet histogram (102).

UTILITY OF PLATELET PARAMETERS TO DISTINGUISH BETWEEN CAUSES OF THROMBOCYTOPENIA

Hypoproductive and peripheral destructive causes of thrombocytopenia:

In our study, we found that platelet counts (PLT-I, PLT-F and smear platelet counts) were significantly lower in patients with hypoproductive thrombocytopenia. Of the platelet parameters studied, IPF, PCT, PDW and P-LCR were all significantly lower in these patients as well. The MPV values did not show any significant difference between the two groups. The median IPF in hypoproductive thrombocytopenia was

6.6% as compared to 15.5% in thrombocytopenia due to peripheral platelet destruction. We found that an IPF cut off of ≥ 9.3% can distinguish between these two groups with a sensitivity of 80% and a specificity of 71%.

Our findings were similar to other studies carried out in this area. Abe *et al.* concluded that IPF was significantly higher in ITP when compared to patients with aplastic anaemia, MDS, leukemia and lymphomas. They suggested an IPF cut off of 7.7% to distinguish ITP from hypo-productive causes of thrombocytopenia with a sensitivity of 88% and specificity of 85.7%. However they also reported that MPV was also significantly higher in patients with ITP (66). Adley *et al.* observed that IPF was significantly higher in patients with ITP when compared to patients with a hematological malignancy. They suggested an IPF cut off of 9.4%, which could pick out patients with ITP with a sensitivity of 86.8% and specificity of 92.6%. They also found that IPF was significantly higher in patients with chronic ITP than in patients with acute ITP. However, our study did not show any statistical significance between these two sub groups, though the IPF was higher in patients with chronic ITP (Median IPF in acute ITP was 16.3% as compared to 22.4% in chronic ITP, p value - 0.12). The lack of statistical significance was probably because of the small patient numbers involved. This study also reported that PDW and P-LCR were significantly higher in patients with thrombocytopenia due to a peripheral destruction of platelets but that PCT was significantly lower in this group of patients when compared to patients with hypo-productive thrombocytopenia (67). This differed from our study since we had found that PCT was also significantly higher in patients with peripheral destruction of platelets. Kickler *et al.* also concluded that IPF was significantly higher in patients

with platelet destruction and that an IPF of > 9% was 100% specific for patients with peripheral platelet destruction (68). Strauss *et al*., Koike *et al.* and Pons *et al.* also reported higher IPF values in patients with ITP than in patients with aplastic anaemia which was statistically significant (69–71). Kaito *et al.* also reported significantly higher values of MPV, PDW and P-LCR in patients with ITP than in patients with aplastic anaemia (97). The lack of a significant difference in MPV between the two groups in our study could be due to differences in the analyser used and due to differences in the different types of diagnoses included under our broad patient groups.

Peripheral destructive causes of thrombocytopenia and ethnic macrothrombocytopenia:

These were the two groups of patients in our study who had higher platelet indices among patients with thrombocytopenia in our study. In clinical practice diagnostic difficulties exist when a patient from North Eastern India presents with thrombocytopenia as both ITP and ethnic macrothrombocytopenia can be asymptomatic (104,105). From our study, we found that platelet counts (PLT-I, PLT-F and smear platelet count) and IPF were all significantly higher in patients with ethnic macrothrombocytopenia than in patients with thrombocytopenia due to peripheral destruction of platelets. The median PLT-F count in patients with ethnic macrothrombocytopenia was 67500/μl as compared to 34000/μl in patients with ITP and hypersplenism. The median IPF in patients with ethnic macrothrombocytopenia was 47.6% as compared to 15.5% in patients with ITP and hypersplenism. Since there

was a clear separation between the median IPF values in patients with ethnic macrothrombocytopenia and in patients with peripheral platelet destruction, we came to the conclusion that an IPF of ≥ 45% could distinguish between these two groups with 100% specificity. Ours is the first study to draw such a conclusion. A similar observation was made by Miyazake *et al*. who reported that the IPF was significantly higher in patients with MYH-9 related giant platelet disorders (Mean - 48.6%) than in patients with ITP (Mean - 18.4%).

PCT, PDW and P-LCR could not be analysed as they were not provided by the analyser in patients with ethnic macrothrombocytopenia due to lack of generation of a platelet histogram from which these parameters are derived. This phenomenon has been reported previously in a study by Bhat *et al.* as well (102).

RELATIONSHIP OF IPF WITH ADEQUACY OF BONE MARROW MEGAKARYOCYTES

To see whether IPF truly does reflect megakaryocytic activity within the bone marrow, we correlated IPF levels independent of patient diagnoses with the presence or absence of megakaryocytes within the bone marrow. We found that the median IPF in patients with adequate or increased bone marrow megakaryocytes was 14.1% which was significantly higher than the median IPF of patients with decreased or dysplastic megakaryocytes (Median - 8.3%). No other studies have compared IPF with the adequacy of bone marrow megakaryocytes. This finding further gives evidence to the currently accepted concept of IPF being a good indicator of bone marrow thrombopoietic activity.

PLATELET MORPHOLOGY IN PATIENTS WITH THROMBOCYTOPENIA

The size of platelets and colour of platelet cytoplasm were assessed on stained peripheral smears of all 198 patients with thrombocytopenia. We found that 30% of circulating platelets in patients with thrombocytopenia due to a peripheral destructive cause were large and very large platelets in comparison to 22% in patients with a decreased production of platelets. In sharp contrast, 59% of circulating platelets in patients with ethnic macrothrombocytopenia were large and very large platelets. These findings do not correlate with our lack of significance in the MPV data between these groups. This is probably due to the fact that in most of our patients, the MPV was obtained from a fitted platelet histogram curve provided on the Beckman Coulter DXH 800 (since Sysmex XN did not provide an MPV in many of these cases) which may not be a true reflection of circulating platelet size. Ingram *et al.* were among the first to describe large platelets in circulation following acute blood loss, initially demonstrated in dogs (106). Ziegler *et al.* also reported that patients with immune thrombocytopenia had larger circulating platelets (9). Noris *et al.* reported that mean platelet diameter (obtained by a software algorithm) was significantly higher in patients with MYH9 mutation associated thrombocytopenia than in patients with immune thrombocytopenia (95). This finding is similar to the much higher number of large platelets seen in our population with ethnic macrothrombocytopenia.

In 2011, a new stage in platelet maturation called pre-platelet (prePLT) was discovered which ranges in size from 2 to 10 μm and are anucleate. PrePLT numbers were found to be high in rats following acute blood loss. It has been demonstrated that one prePLT can divide to form 2 normal sized platelets. They are being equated to

young newly released platelets as well as the large platelets seen in inherited macrothrombocytopenia (4,107). Our finding of the presence of more larger sized platelets in patients with thrombocytopenia due to peripheral platelet destruction and more so in patients with ethnic macrothrombocytopenia could be explained by this phenomenon of prePLT release into the circulation by megakaryocytes due to accelerated platelet turnover or some platelet cytoskeletal abnormality (in ethnic macrothrombocytopenia subgroup) in these patients.

Our assessment of platelet cytoplasmic colour showed that 87% of patients with a peripheral destructive cause of thrombocytopenia had more bluish tinged (more basophilic) platelets in circulation. The remaining 13% of platelets were only weakly basophilic with a more greyish appearance to the cytoplasm which is the normal cytoplasmic colour of platelets (7). 69% of patients with thrombocytopenia due to decreased platelet production, had more bluish platelets in circulation and 24% of them had predominantly greyish platelets. The difference in cytoplasmic colour was much more evident in patients with ethnic macrothrombocytopenia, 92% of whom had more basophilic circulating platelets. However, this difference in cytoplasmic colour between the three different cohorts was not statistically significant.

Cytoplasmic colouration which is a function of staining and cytoplasmic content, varies from cell to cell based on the pH of the cytoplasm. The modified Wright-Giemsa stain used in our study is a Romanowsky stain which has a basic dye which gives acidic substances a bluish colour and an acidic dye which gives basic substances a reddish or orange colour. Nucleic acids like RNA and DNA are acidic substances and hence take up the basic dye thus imparting a basophilic colour to any cell

structure that contains them. So a cytoplasm which is rich in RNA will be more bluish in colour (7). We know that newly released platelets in circulation have more RNA within their cytoplasm which could either be due to their larger size or due to their younger age (as RNA is lost with passage of time) or a combination of these two factors. Hence it can be hypothesized that these immature platelets when seen on a Romanowsky dye stained peripheral smear will have a more bluish tinge to the cytoplasm than the normally present circulating mature platelets. This hypothesis is supported by our findings in the assessment of platelet cytoplasmic colour. Since patients with peripheral destruction of platelets have a normally functioning bone marrow, they are able to compensate for the thrombocytopenia by producing platelets more effectively which is not so in patients with a hypoproductive cause of thrombocytopenia where the marrow response is blunted. This is probably why the former group had more bluish platelets in circulation.

Patients with ethnic macrothrombocytopenia have very large platelets as evidenced by our morphological study of platelet size. It is possible that these large platelets have more RNA within their cytoplasm simply because of their large size or due to some alteration from normal in their production from megakaryocytes which is yet to be elucidated. This could be the reason behind our finding that the majority of these individuals had very basophilic platelets. This theory is also supported by our finding that patients with ethnic macrothrombocytopenia had the highest IPF values among all the patients in our study since IPF is a function of platelet size as well as platelet cytoplasmic RNA content. There have not been any studies so far which have studied platelet cytoplasmic colour in correlation with IPF or aetiology of thrombocytopenia.

TRENDS OF IPF IN PATIENTS POST HEMATOPOIETIC STEM CELL TRANSPLANT

Since IPF appears to be an indicator that has potential to predict the state of the bone marrow, we have attempted to utilize it as a marker of platelet recovery in different groups of patients who have undergone allogenic and autologous bone marrow stem cell transplantation for different haematological conditions specified below.

Patient demographics: We followed up a total of 43 patients who underwent hematopoietic stem cell transplantation (HSCT). There were an almost equal number of children (51%) and adults in our study group and the ages ranged from 2 years to 59 years. There was a slight male predominance with 63% of patients being males and only 37% females.

Disease subgroups: Out of the 43 patients, 19 patients had aplastic anaemia, 11 patients had beta thalassemia major, 9 patients had multiple myeloma and 4 patients had lymphoma (2 patients with Hodgkin lymphoma, 1 patient with Diffuse large B cell lymphoma and 1 patient with Angioimmunoblastic T cell lymphoma). 2 of these patients were undergoing a second transplant after a previous engraftment failure. The patients with Aplastic anaemia and Thalassemia underwent Allogenic HSCT (30 patients) and the patients with Myeloma and Lymphoma (13 patients) underwent Autologous HSCT.

HLA cross match status and Conditioning regimens used: 21 out of 30 patients who underwent Allogenic HSCT had a fully HLA cross matched sibling donor transplant. 3 patients had a fully matched family donor, 1 patient had a fully matched unrelated donor, 3 patients had a haplomatched donor and 2 patients had 2 antigen mismatched donors. 60% of patients who underwent Allogenic HSCT had a non-myeloablative conditioning regimen and the remaining 40% of patients had a myeloablative conditioning regimen. All patients with myeloma and lymphoma who underwent autologous HSCT were conditioned with Melphalan and BEAM protocol (Carmustine, Etoposide, Cytarabine and Melphalan) respectively.

TREND OF IPF IN PATIENTS WITH APLASTIC ANAEMIA AND THALSSEMIA POST ALLOGENIC HSCT

We followed up these patients with serial measurements of IPF from Day 0 of transplant (i.e. day of infusion of peripheral blood stem cells) up until the day of platelet recovery (which we defined as the third day of an unsupported platelet count of >20000/μl) and beyond. The median day of platelet recovery in patients with Aplastic anaemia was 12 days and that for patients with Thalassemia was 13 days.

Aplastic anaemia: On applying the upper limit of our reference interval for IPF as a cut off, in 5 out of 7 adult patients and 8 out of 9 children, IPF went above the upper limit of reference intervals for the respective groups either a few days before or on the day of platelet recovery. In children, the IPF went above the reference range of 3.1% a median of 1.5 days before platelet recovery. In adults, the IPF went above the reference range of 6.1% a median of 1 day before platelet recovery. In children the

interval by which the IPF preceded platelet recovery was found to range from 0 days (i.e. on the day of platelet recovery) to 16 days and in adults, this ranged from on the day of platelet recovery to 12 days before recovery.

In total, this showed that in 56% of patients who engrafted, IPF showed an early indication of impending increase in platelet count. In 25% of patients, IPF went above reference interval on the day of platelet recovery. 2 patients did not show an increase in IPF above the reference interval even though they engrafted and in one patient, the IPF was always above the normal reference interval, probably because this patient was from Jharkhand and had an added component of constitutional macrothrombocytopenia contributing to the high IPF values.

Thalassemia: All patients in this subgroup were children and we found that the IPF values of all patients went above the reference range (3.1%) a median of 3.5 days (ranging from 0 to 12 days) before platelet recovery.

Morkis *et al.* reported that IPF values of >6.2% predicted platelet engraftment within a few days in 92% of patients who underwent autologous and allogenic HSCT. The cut off of 6.2% was chosen because it was the upper limit of normal reference interval for the parameter. However, their criteria for platelet engraftment was different from ours in that they had taken the "first of three days with an unsupported platelet count of > 20000/μl" as the day of platelet engraftment. They also observed that IPF showed a lot of variations when serially followed up in patients post allogenic HSCT (77).

Goncalo *et al.* recorded serial IPF in 46 adults who underwent allogenic HSCT and observed that there was a rise in IPF > 7% a median of 2 days before platelet

recovery. They had taken platelet recovery as the "first of seven days with an unsupported platelet count of > 20000/μl" (78). Takami *et al.* in 6 patients post allogenic HSCT reported that an IPF rise > 3% occurs one day before platelet engraftment which they defined as "platelet count of > 30000/μl for 3 consecutive measurements on different days in the absence of platelet transfusions for at least 7 days" (79). Briggs *et al.* in a study on 7 patients post allogenic HSCT concluded that there was a rise in IPF around 1-3 days before platelet recovery (no definite definition for this was provided) and that there were variations in IPF during the course of follow up. They attributed this variation to platelet transfusions stating that IPF values fell for 24 hours after a platelet transfusion. They had suggested that this could be due to a suppression of thrombopoiesis due to the transfusion (82). However, in our study, we did not find any consistent change in IPF values after platelet transfusions. Yamaoka *et al.* using a platelet recovery definition as "a platelet count > 100000/μl for 3 consecutive days without platelet transfusions" concluded that patients post allogenic transplant (11 patients) with a peak IPF > 10% achieved platelet recovery a median of 2 days later (83).

TREND OF IPF IN PATIENTS WITH MULTIPLE MYELOMA AND LYMPHOMA POST AUTOLOGOUS HSCT

We followed up these patients with serial measurements of IPF from Day 0 of transplant (i.e. day of infusion of peripheral blood stem cells) up until the day of platelet recovery (which we defined as the third day of an unsupported platelet count of >20000/μl) and beyond. Most of these patients had IPF values being recorded only

every alternate day as we did not want to interfere with the follow up protocols that are in place at our institution. The median day of platelet recovery in patients with Multiple myeloma was 12 days and that for patients with Lymphoma was 15 days.

Since all these patients were adults, we applied a cut off of 6.1% and found that the IPF in 9 out of the 13 patients studied (69% of patients) went above this value a median of 3 days before platelet recovery (ranged from 0 - 10 days). 2 patients did not show an increase in IPF above the reference interval even though they engrafted and in a further 2 patients, the IPF was always above the normal reference interval, probably because these patients were from North East Indian states and had an added component of constitutional macrothrombocytopenia contributing to the high IPF values.

Van der Linden *et al.* in their study on 16 patients undergoing autologous transplant, defined platelet recovery as "an increase in platelets greater than its reference change value and not due to platelet transfusion" and concluded that an IPF cut off of 5.3% could predict platelet recovery within 2 days. They also did not find any consistent relationship between platelet transfusion and change in IPF levels (80). Briggs *et al.* in a study on 8 autologous transplant patients concluded that there was a mean rise in IPF of 12.8% which preceded platelet recovery (not defined) by 1 to 2 days (82).

From the above discussion, it is clear that a rise in IPF precedes platelet recovery by 1 - 3 days in patients post allogenic or autologous HSCT. Our study also supports this view. The fact that different authors have used different definitions for platelet recovery and different cut-offs for IPF rise is probably the reason why we are not left

with a definite IPF cut off or a definite predictive time interval after IPF rise within which we can expect to see platelet recovery. Our study also supports the observations made by a few authors on the high variability of IPF in patients undergoing allogenic HSCT. This has been attributed to transient rises in IPF seen due to sepsis which is common in this patient group due to profound immunosuppression (82). Another reason why there is a difference in the number of days from rise in IPF to platelet recovery could be the fact that all these studies included a variety of patient groups (including patients with hematological malignancies and myelodysplastic syndrome) undergoing allogenic transplant and was not just limited to Aplastic anaemia and Thalassemia patients as was the case in our study. The bone marrow kinetics is different in different disease processes and hence may also influence recovery in different ways in the post-transplant period.

TREND OF IPF IN PATIENTS WITH PRIMARY ENGRAFTMENT FAILURE

Primary engraftment failure was defined as "lack of absolute neutrophil count to reach $\geq 0.5 \times 10^9$/L" (108). We had 3 patients with aplastic anaemia who underwent allogenic HSCT in our study who were diagnosed with primary graft failure. One patient was an adult and the other 2 were children. When we looked at the serial IPF values in these patients, we found that the IPF value never went > 6.1% in the adult patient. In the two children, IPF did go > 3.1%, but only on day 22-23 which was much later than all the other engrafted patients. Another trend that we noticed was that there was no consistent rise in IPF values and that there were large variations in the daily IPF being recorded. This finding is probably because the marrow is unable to

produce platelets due to lack of donor cell engraftment. This is the first time that the trend of IPF is being looked at in patients with primary engraftment failure and we could not find any literature to compare our findings with.

In conclusion, from our analysis of 43 patients who underwent allogenic and autologous HSCT, we found that the IPF went above the age specific reference interval cut offs in 33 patients (76%) and that this rise preceded platelet recovery by a median of 1 - 3.5 days. This time to platelet recovery differed based on the disease condition that the patient had being shortest in patients with aplastic anaemia post allogenic HSCT and longest in patients with thalassemia post allogenic HSCT. This could be explained by the co-existent hypersplenism that 7 out of 11 patients in our study had and the possibility that all the platelets that are being produced in the marrow are being sequestered in the spleen leading to a longer time to platelet recovery. We were unable to provide a definite cut-off for IPF which could predict platelet engraftment due to the low patient numbers involved in the study and due to the marked day to day variation in IPF in these patients. Hence, we took the upper limit of the normal reference interval as our reference point. There is a wide variation in the IPF cut offs and definitions of platelet recovery that have been used from study to study and this is what has led to different results. In patients with primary engraftment failure, we found that there were marked daily variations in IPF and that there was never a sustained rising trend of IPF in these patients as was seen in others. Also, in 1 of these patients IPF never went above age specific reference interval limit.

With these findings, we are able to say that IPF (> 3.1% in children and > 6.1% in adults) can probably be used as a marker to predict platelet recovery in patients post HSCT and that serial monitoring of IPF with its trend analysis could also be a predictor of primary engraftment failure.

From our analysis of 196 patients with thrombocytopenia, we found that the IPF in patients with a hypoproductive or peripheral destructive cause of thrombocytopenia is significantly higher than that of the normal control population. We also found that patients with thrombocytopenia due to peripheral platelet destruction have a significantly higher IPF than patients with decreased platelet production and that an IPF of ≥ 9.3% can distinguish between patients with thrombocytopenia due to peripheral destruction of platelets from patients with thrombocytopenia due to decreased production of platelets with a sensitivity of 80% and a specificity of 71%. In addition, an IPF of ≥ 45% can distinguish between thrombocytopenia due to peripheral destruction of platelets from ethnic macrothrombocytopenia with 100% specificity. Individuals with ethnic macrothrombocytopenia had the highest IPF values amongst all the patient groups studied. Interestingly, these individuals also had the highest proportion of large platelets with blue cytoplasm found on peripheral smear examination. Another noteworthy finding was that the IPF was significantly higher in patients with adequate and increased bone marrow megakaryocytes when compared to patients with decreased and absent bone marrow megakaryocytes. This could be evidence to support the current understanding of IPF as a marker of thrombopoietic activity of the bone marrow.

LIMITATIONS OF THE STUDY

- Reference range calculation for the Indian population had to be done after excluding individuals coming from North East Indian states because of the high incidence of constitutional macrothrombocytopenia and resultant high values of IPF.

- As it was a prospective study, the number of patients that could be enrolled for follow up after hematopoietic stem cell transplant was limited by the duration of the study period. Derivation of an absolute value of IPF or a percentage rise in IPF from baseline which would predict platelet recovery needs a larger cohort of patients and a more varied group of primary disease conditions so that we can observe differences if any, in patterns of IPF depending on the reason for HSCT.

- We did not interfere with existing platelet transfusion protocols for patients post HSCT, and hence were unable to test whether our proposed time intervals from IPF rise till platelet recovery held true even without platelet transfusion support.

- We had an interesting observation in the trend of IPF in patients with primary engraftment failure which has not been reported previously before, but further confirmation of this finding requires the follow up of more such patients.

CONCLUSIONS

- Patients with thrombocytopenia due to peripheral platelet destruction have a significantly higher IPF than patients with thrombocytopenia due to decreased platelet production.

- A subgroup of individuals from the North Eastern states of India have an asymptomatic form of thrombocytopenia called ethnic macrothrombocytopenia characterized by the presence of large blue platelets in peripheral smear and the highest IPF among the patient groups studied.

- An IPF of ≥ 9.3% can distinguish between patients with thrombocytopenia due to peripheral destruction of platelets from patients with thrombocytopenia due to decreased production of platelets with a sensitivity of 80% and a specificity of 71%.

- An IPF of ≥ 45% can distinguish between thrombocytopenia due to peripheral destruction of platelets from ethnic macrothrombocytopenia with 100% specificity.

- IPF was significantly higher in patients with adequate and increased bone marrow megakaryocytes when compared to patients with decreased and absent bone marrow megakaryocytes.

- In majority of patients (76%) being followed up post HSCT, IPF went above the age specific reference interval cut offs and this rise preceded platelet recovery by a median of 1 - 3.5 days.

- The time to platelet recovery after rise in IPF differed based on the disease condition that the patient had and the type of transplant (autologous or allogenic) being shortest in patients with aplastic anaemia post allogenic HSCT and longest in patients with thalassemia post allogenic HSCT and in patients post autologous stem cell transplant.

- Patients with primary engraftment failure had a marked daily variation in IPF levels with no sustained rise in IPF and in one out of the three patients studied, the IPF did not go above the age specific reference interval cut off.

With these findings, we conclude that IPF may be a reliable marker of thrombopoietic activity within the marrow and that it can be used as marker to distinguish between thrombocytopenia due to peripheral platelet destruction from thrombocytopenia due to decreased platelet production and ethnic macrothrombocytopenia. It may also have potential utility in the monitoring of patients post hematopoietic stem cell transplantation to predict platelet recovery and thereby curb unnecessary platelet transfusions in these patients. From our study we also find that analyzing the pattern of IPF could be even used to predict primary engraftment failure in this patient group.

BIBLIOGRAPHY

1. Brewer DB. Max Schultze (1865), G. Bizzozero (1882) and the discovery of the platelet. Br J Haematol. 2006 May;133(3):251–8.

2. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. J Cell Biol. 2013 Jun 10;201(6):785–96.

3. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005 Dec 1;115(12):3339–47.

4. Machlus KR, Thon JN, Italiano JE. Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. Br J Haematol. 2014 Apr;165(2):227–36.

5. Thon JN, Montalvo A, Patel-Hett S, Devine MT, Richardson JL, Ehrlicher A, et al. Cytoskeletal mechanics of proplatelet maturation and platelet release. J Cell Biol. 2010 Nov 15;191(4):861–74.

6. Bain BJ, Lewis SM, Dacie JV. Dacie and Lewis practical haematology [Internet]. [Edinburgh]: Elsevier Churchill Livingstone; 2012 [cited 2015 Dec 13]. Available from: http://www.clinicalkey.com/dura/browse/bookChapter/3-s2.0-C20090316293

7. Bain BJ. Blood cells: a practical guide [Internet]. John Wiley & Sons; 2015 [cited 2015 Dec 13].

8. Hirsh J. Platelet age: its relationship to platelet size, function and metabolism. Br J Haematol. 1972;23(s1):209–214.

9. Zeigler Z, Murphy S, Gardner FH. Microscopic platelet size and morphology in various hematologic disorders. Blood. 1978 Mar;51(3):479–86.

10. Mhawech P, Saleem A. Inherited giant platelet disorders classification and literature review. Am J Clin Pathol. 2000;113(2):176–190.

11. Stasi R. How to approach thrombocytopenia. ASH Educ Program Book. 2012;2012(1):191–197.

12. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009 Mar 12;113(11):2386–93.

13. Smock KJ, Perkins SL. Thrombocytopenia: an update. Int J Lab Hematol. 2014 Jun;36(3):269–78.

14. Drachman JG. Inherited thrombocytopenia: when a low platelet count does not mean ITP. Blood. 2004 Jan 15;103(2):390–8.

15. Visentin GP, Liu CY. Drug-induced thrombocytopenia. Hematol Oncol Clin North Am. 2007 Aug;21(4):685–696, vi.

16. Brecher G, Schneiderman M, Cronkite EP. The reproducibility and constancy of the platelet count. Am J Clin Pathol. 1953 Jan;23(1):15–26.

17. Briggs C, Harrison P, Machin SJ. Continuing developments with the automated platelet count1: DEVELOPMENTS WITH THE AUTOMATED PLATELET COUNT. Int J Lab Hematol. 2007 Apr;29(2):77–91.

18. Harrison P, Ault KA, Chapman S, Charie L, Davis B, Fujimoto K, et al. An interlaboratory study of a candidate reference method for platelet counting. Am J Clin Pathol. 2001;115(3):448–459.

19. Webb DI, Parker L, Webb K. Platelet count assessment from peripheral blood smear (PBS). Alaska Med. 2004 Dec;46(4):92–5.

20. Malok M, Titchener EH, Bridgers C, Lee BY, Bamberg R. Comparison of two platelet count estimation methodologies for peripheral blood smears. Clin Lab Sci J Am Soc Med Technol. 2007;20(3):154–60.

21. Simson E. Wallace Coulter's life and his impact on the world. Int J Lab Hematol. 2013 Jun;35(3):230–6.

22. Zandecki M, Genevieve F, Gerard J, Godon A. Spurious counts and spurious results on haematology analysers: a review. Part I: platelets. Clin Lab Haematol. 2007 Feb;29(1):4–20.

23. Briggs C, Harrison P, Grant D, Staves J, MacHin SJ. New quantitative parameters on a recently introduced automated blood cell counter–the XE 2100TM. Clin Lab Haematol. 2000;22(6):345–350.

24. Segal HC, Briggs C, Kunka S, Casbard A, Harrison P, Machin SJ, et al. Accuracy of platelet counting haematology analysers in severe thrombocytopenia and potential impact on platelet transfusion. Br J Haematol. 2005 Feb;128(4):520–5.

25. Briggs C, Kunka S, Machin SJ. The most accurate platelet count on the Sysmex XE-2100. Optical or impedance? Clin Lab Haematol. 2004;26(2):157–158.

26. Hedley BD, Keeney M, Chin-Yee I, Brown W. Initial performance evaluation of the UniCel® DxH 800 Coulter® cellular analysis system: DXH 800 PERFORMANCE EVALUATION. Int J Lab Hematol. 2011 Feb;33(1):45–56.

27. Briggs C, Longair I, Kumar P, Singh D, Machin SJ. Performance evaluation of the Sysmex haematology XN modular system. J Clin Pathol. 2012 Nov 1;65(11):1024–30.

28. Tanaka Y, Tanaka Y, Gondo K, Maruki Y, Kondo T, Asai S, et al. Performance Evaluation of Platelet Counting by Novel Fluorescent Dye Staining in the XN-Series Automated Hematology Analyzers: Platelet Counting by Fluorescent Dye Staining. J Clin Lab Anal. 2014 Sep;28(5):341–8.

29. Park SH, Park C-J, Kim M-J, Han M-Y, Lee B-R, Cho Y-U, et al. The Sysmex XN-2000 Hematology Autoanalyzer Provides a Highly Accurate Platelet Count than the Former Sysmex XE-2100 System Based on Comparison with the CD41/CD61 Immunoplatelet Reference Method of Flow Cytometry. Ann Lab Med. 2014;34(6):471.

30. Schoorl M, Schoorl M, Oomes J, van Pelt J. New Fluorescent Method (PLT-F) on Sysmex XN2000 Hematology Analyzer Achieved Higher Accuracy in Low Platelet Counting. Am J Clin Pathol. 2013 Oct 1;140(4):495–9.

31. D'Souza C, Briggs C, Machin SJ. Platelets. Clin Lab Med. 2015 Mar;35(1):123–31.

32. Harrison P, Goodall AH. "Message in the Platelet" – more than just vestigial mRNA! Platelets. 2008 Jan;19(6):395–404.

33. Rowley JW, Schwertz H, Weyrich AS. Platelet mRNA: the meaning behind the message. Curr Opin Hematol. 2012 Sep;19(5):385–91.

34. Garg SK, Amorosi EL, Karpatkin S. Use of the megathrombocyte as an index of megakaryocyte number. N Engl J Med. 1971 Jan 7;284(1):11–7.

35. Detwiler TC, Odell TT, MacDONALD TP. Platelet size, ATP content, and clot retraction in relation to platelet age. Am J Physiol. 1962 Jul;203:107–10.

36. Mcdonald TP, Odell TT, Gosslee DG. PLATELET SIZE IN RELATION TO PLATELET AGE. Proc Soc Exp Biol Med Soc Exp Biol Med N Y N. 1964 Mar;115:684–9.

37. Shulman NR, Watkins SP, Itscoitz SB, Students AB. Evidence that the spleen retains the youngest and hemostatically most effective platelets. Trans Assoc Am Physicians. 1968;81:302–13.

38. Ault KA, Knowles C. In vivo biotinylation demonstrates that reticulated platelets are the youngest platelets in circulation. Exp Hematol. 1995 Aug;23(9):996–1001.

39. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood. 1990 Jan 1;75(1):116–21.

40. van den Broek PhD NM. Reference intervals of reticulated platelets and other platelet parameters and their associations. Arch Pathol Lab Med. 2013;137(11):1635.

41. HARRISON P. Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. Br J Haematol. 1998;100:351–357.

42. Hoffmann JJML. Reticulated platelets: analytical aspects and clinical utility. Clin Chem Lab Med CCLM [Internet]. 2014 Jan 1 [cited 2015 Dec 7];52(8). Available from: http://www.degruyter.com/view/j/cclm.2014.52.issue-8/cclm-2014-0165/cclm-2014-0165.xml

43. Rinder HM, Munz UJ, Ault KA, Bonan JL, Smith BR. Reticulated platelets in the evaluation of thrombopoietic disorders. Arch Pathol Lab Med. 1993 Jun;117(6):606–10.

44. Salvagno GL, Montagnana M, Degan M, Marradi PL, Ricetti MM, Riolfi P, et al. Evaluation of platelet turnover by flow cytometry. Platelets. 2006 Jan;17(3):170–7.

45. Ogata H. Measurement of reticulated platelets in thrombocytopenia--methodology and clinical utility. Kurume Med J. 1998;45(2):165–70.

46. Jiménez MM, Guedán MJA, Martín LM, Campos JAS, Martínez IR, Vilella CT. Measurement of reticulated platelets by simple flow cytometry: An indirect thrombocytopoietic marker. Eur J Intern Med. 2006 Dec;17(8):541–4.

47. Wang C, Smith BR, Ault KA, Rinder HM. Reticulated platelets predict platelet count recovery following chemotherapy. Transfusion (Paris). 2002 Mar 1;42(3):368–74.

48. Chaoui D, Chakroun T, Robert F, Rio B, Belhocine R, Legrand O, et al. Reticulated platelets: a reliable measure to reduce prophylactic platelet transfusions after intensive chemotherapy. Transfusion (Paris). 2005 May;45(5):766–72.

49. Michur H, Maślanka K, Szczepiński A, Mariańska B. Reticulated platelets as a marker of platelet recovery after allogeneic stem cell transplantation. Int J Lab Hematol. 2008 Dec 1;30(6):519–25.

50. Romp KG, Peters WP, Hoffman M. Reticulated platelet counts in patients undergoing autologous bone marrow transplantation: an aid in assessing marrow recovery. Am J Hematol. 1994 Aug;46(4):319–24.

51. Rinder HM, Bonan JL, Anandan S, Rinder CS, Rodrigues PA, Smith BR. Noninvasive measurement of platelet kinetics in normal and hypertensive pregnancies. Am J Obstet Gynecol. 1994 Jan;170(1 Pt 1):117–22.

52. Rinder HM, Schuster JE, Rinder CS, Wang C, Schweidler HJ, Smith BR. Correlation of thrombosis with increased platelet turnover in thrombocytosis. Blood. 1998 Feb 15;91(4):1288–94.

53. Dusse LMS, Freitas LG. Clinical applicability of reticulated platelets. Clin Chim Acta. 2015 Jan;439:143–7.

54. Noronha JFA, Costa FF, Saad STO, Lorand-Metze IGH, Grotto HZW. Evaluation of reticulated platelets in patients with sickle cell diseases. Thromb Res. 2007;121(2):259–67.

55. Himmelfarb J, Holbrook D, McMonagle E, Ault K. Increased reticulated platelets in dialysis patients. Kidney Int. 1997 Mar;51(3):834–9.

56. De Blasi RA, Cardelli P, Costante A, Sandri M, Mercieri M, Arcioni R. Immature platelet fraction in predicting sepsis in critically ill patients. Intensive Care Med. 2013 Apr;39(4):636–43.

57. Watanabe K, Kawai Y, Takeuchi K. [Reticulated platelets--automated measurement and clinical utility]. Rinshō Ketsueki Jpn J Clin Hematol. 1995 Apr;36(4):267–72.

58. Garibaldi B, Malani R, Yeh H-C, Lipson E, Michell D, Bennett M, et al. Estimating platelet production in patients with HIV-related thrombocytopenia using the immature platelet fraction. Am J Hematol. 2009 Dec;84(12):852–4.

59. Briggs C, Kunka S, Hart D, Oguni S, Machin SJ. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. Br J Haematol. 2004 Jul;126(1):93–9.

60. Osei-Bimpong A, Saleh M, Sola-Visner M, Widness J, Veng-Pedersen P. Correction for effect of cold storage on immature platelet fraction. J Clin Lab Anal. 2010;24(6):431–3.

61. Nomura T, Kubota Y, Kitanaka A, Kurokouchi K, Inage T, Saigo K, et al. Immature platelet fraction measurement in patients with chronic liver disease: a convenient marker for evaluating cirrhotic change. Int J Lab Hematol. 2010 Jun;32(3):299–306.

62. Dadu T, Sehgal K, Joshi M, Khodaiji S. Evaluation of the immature platelet fraction as an indicator of platelet recovery in dengue patients. Int J Lab Hematol. 2014 Oct;36(5):499–504.

63. Osei-Bimpong A. The effect of storage on the clinical utility of the immature platelet fraction. Hematology. 2009 Apr;14(2):118–21.

64. Grillone R, Grimaldi E, Scopacasa F, Conticelli M, Dente B. Evaluation of the reticulocyte counting by the Mindray BC 6800 automated hematology analyzer: comparison with ABX Pentra 120, Coulter LH 750, and microscopy. Int J Lab Hematol. 2015 Feb 1;37(1):e3–6.

65. Meintker L, Haimerl M, Ringwald J, Krause SW. Measurement of immature platelets with Abbott CD-Sapphire and Sysmex XE-5000 in haematology and

oncology patients. Clin Chem Lab Med [Internet]. 2013 Jan 1 [cited 2015 Dec 31];51(11). Available from: http://www.degruyter.com/view/j/cclm.2013.51.issue-11/cclm-2013-0252/cclm-2013-0252.xml

66. Abe Y, Wada H, Tomatsu H, Sakaguchi A, Nishioka J, Yabu Y, et al. A simple technique to determine thrombopoiesis level using immature platelet fraction (IPF). Thromb Res. 2006 Jan;118(4):463–9.

67. Adly AAM, Ragab IA, Ismail EAR, Farahat MM. Evaluation of the immature platelet fraction in the diagnosis and prognosis of childhood immune thrombocytopenia. Platelets. 2015 Oct 3;26(7):645–50.

68. Kickler TS, Oguni S, Borowitz MJ. A Clinical Evaluation of High Fluorescent Platelet Fraction Percentage in Thrombocytopenia. Am J Clin Pathol. 2006 Feb;125(2):282–7.

69. Strauß G, Vollert C, von Stackelberg A, Weimann A, Gaedicke G, Schulze H. Immature platelet count: A simple parameter for distinguishing thrombocytopenia in pediatric acute lymphocytic leukemia from immune thrombocytopenia. Pediatr Blood Cancer. 2011 Oct;57(4):641–7.

70. Koike Y, Miyazaki K, Higashihara M, Kimura E, Jona M, Uchihashi K, et al. Clinical significance of detection of immature platelets: comparison between percentage of reticulated platelets as detected by flow cytometry and immature platelet fraction as detected by automated measurement. Eur J Haematol. 2010 Feb;84(2):183–4.

71. Pons I, Monteagudo M, Lucchetti G, Muñoz L, Perea G, Colomina I, et al. CORRELATION BETWEEN IMMATURE PLATELET FRACTION AND RETICULATED PLATELETS. USEFULNESS IN THE AETIOLOGY DIAGNOSIS OF THROMBOCYTOPENIA. Eur J Haematol. 2010 May;no-no.

72. Miyazaki K, Koike Y, Kunishima S, Ishii R, Danbara M, Horie R, et al. Immature platelet fraction measurement is influenced by platelet size and is a useful parameter for discrimination of macrothrombocytopenia. Hematology. 2015 Dec;20(10):587–92.

73. Chang Y-J, Xu L-P, Liu D-H, Liu K-Y, Han W, Chen Y-H, et al. Platelet Engraftment in Patients with Hematologic Malignancies following Unmanipulated Haploidentical Blood and Marrow Transplantation: Effects of CD34+ Cell Dose and Disease Status. Biol Blood Marrow Transplant. 2009 May;15(5):632–8.

74. Mackall C, Fry T, Gress R, Peggs K, Storek J, Toubert A. Background to hematopoietic cell transplantation, including post transplant immune recovery. Bone Marrow Transplant. 2009 Oct;44(8):457–62.

75. Grazziutti ML, Dong L, Miceli MH, Cottler-Fox M, Krishna SG, Fassas A, et al. Recovery from neutropenia can be predicted by the immature reticulocyte fraction several days before neutrophil recovery in autologous stem cell transplant recipients. Bone Marrow Transplant. 2006;37(4):403–409.

76. Annen K, Olson JE. Optimizing platelet transfusions: Curr Opin Hematol. 2015 Sep;1.

77. Morkis IVC, Farias MG, Rigoni LDC, Scotti L, Gregianin LJ, Daudt LE, et al. Assessment of immature platelet fraction and immature reticulocyte fraction as predictors of engraftment after hematopoietic stem cell transplantation. Int J Lab Hematol. 2015 Apr;37(2):259–64.

78. Gonçalo AP, Barbosa IL, Campilho F, Campos A, Mendes C. Predictive Value of Immature Reticulocyte and Platelet Fractions in Hematopoietic Recovery of Allograft Patients. Transplant Proc. 2011 Jan;43(1):241–3.

79. Takami A, Shibayama M, Orito M, Omote M, Okumura H, Yamashita T, et al. Immature platelet fraction for prediction of platelet engraftment after allogeneic stem cell transplantation. Bone Marrow Transplant. 2007 Apr;39(8):501–7.

80. van der Linden N, Klinkenberg LJJ, Meex SJR, Beckers EAM, de Wit NCJ, Prinzen L. Immature platelet fraction measured on the Sysmex XN hemocytometer predicts thrombopoietic recovery after autologous stem cell transplantation. Eur J Haematol. 2014 Aug;93(2):150–6.

81. Bat T, Leitman SF, Calvo KR, Chauvet D, Dunbar CE. Measurement of the absolute immature platelet number reflects marrow production and is not impacted by platelet transfusion: Recent PLT Transfusion and Immature PLT Variables. Transfusion (Paris). 2013 Jun;53(6):1201–4.

82. Briggs C, Hart D, Kunka S, Oguni S, Machin SJ. Immature platelet fraction measurement: a future guide to platelet transfusion requirement after haematopoietic stem cell transplantation. Transfus Med. 2006 Apr;16(2):101–9.

83. Yamaoka G, Kubota Y, Nomura T, Inage T, Arai T, Kitanaka A, et al. The immature platelet fraction is a useful marker for predicting the timing of platelet recovery in patients with cancer after chemotherapy and hematopoietic stem cell transplantation: PREDICTION OF PLATELET RECOVERY BY IPF. Int J Lab Hematol. 2010 Dec;32(6p1):e208–16.

84. Saigo K, Sakota Y, Masuda Y, Matsunaga K, Takenokuchi M, Nishimura K, et al. Automatic detection of immature platelets for decision making regarding platelet transfusion indications for pediatric patients. Transfus Apher Sci Off J World Apher Assoc Off J Eur Soc Haemapheresis. 2008 Apr;38(2):127–32.

85. Have LWJ, Hasle H, Vestergaard EM, Kjaersgaard M. Absolute immature platelet count may predict imminent platelet recovery in thrombocytopenic

children following chemotherapy. Pediatr Blood Cancer. 2013 Jul;60(7):1198–203.

86. Lecompte TP, Bernimoulin MP. Novel Parameters in Blood Cell Counters. Clin Lab Med. 2015 Mar;35(1):209–24.

87. Briggs C. Quality counts: new parameters in blood cell counting. Int J Lab Hematol. 2009 Jun;31(3):277–97.

88. Rodak BF, Keohane EM, Walenga JM, Smith LJ. Rodak's hematology: clinical principles and applications. 2016.

89. Bowles KM, Cooke LJ, Richards EM, Baglin TP. Platelet size has diagnostic predictive value in patients with thrombocytopenia. Clin Lab Haematol. 2005;27(6):370–373.

90. Vasse M, Masure A, Lenormand B. Mean platelet volume is highly correlated to platelet count. Thromb Res. 2012 Sep;130(3):559–60.

91. Giles C. The platelet count and mean platelet volume. Br J Haematol. 1981 May;48(1):31–7.

92. Ntaios G, Papadopoulos A, Chatzinikolaou A, Saouli Z, Karalazou P, Kaiafa G, et al. Increased Values of Mean Platelet Volume and Platelet Size Deviation Width May Provide a Safe Positive Diagnosis of Idiopathic Thrombocytopenic Purpura. Acta Haematol. 2008;119(3):173–7.

93. Numbenjapon T, Mahapo N, Pornvipavee R, Sriswasdi C, Mongkonsritragoon W, Leelasiri A, et al. A prospective evaluation of normal mean platelet volume in discriminating hyperdestructive thrombocytopenia from hypoproductive thrombocytopenia. Int J Lab Hematol. 2008 Oct;30(5):408–14.

94. Noris P, Klersy C, Zecca M, Arcaini L, Pecci A, Melazzini F, et al. Platelet size distinguishes between inherited macrothrombocytopenias and immune thrombocytopenia. J Thromb Haemost. 2009 Dec;7(12):2131–6.

95. Noris P, Klersy C, Gresele P, Giona F, Giordano P, Minuz P, et al. Platelet size for distinguishing between inherited thrombocytopenias and immune thrombocytopenia: a multicentric, real life study. Br J Haematol. 2013 Jul;162(1):112–9.

96. Kim MJ, Park P-W, Seo Y-H, Kim K-H, Seo JY, Jeong J-H, et al. Comparison of platelet parameters in thrombocytopenic patients associated with acute myeloid leukemia and primary immune thrombocytopenia: Blood Coagul Fibrinolysis. 2014 Apr;25(3):221–5.

97. Kaito K, Otsubo H, Usui N, Yoshida M, Tanno J, Kurihara E, et al. Platelet size deviation width, platelet large cell ratio, and mean platelet volume have

sufficient sensitivity and specificity in the diagnosis of immune thrombocytopenia. Br J Haematol. 2005 Mar;128(5):698–702.

98. Ko YJ, Hur M, Kim H, Choi SG, Moon H-W, Yun Y-M. Reference interval for immature platelet fraction on Sysmex XN hematology analyzer: a comparison study with Sysmex XE-2100. Clin Chem Lab Med CCLM [Internet]. 2015 Jan 1 [cited 2016 Jul 25];53(7). Available from: http://www.degruyter.com/view/j/cclm.2015.53.issue-7/cclm-2014-0839/cclm-2014-0839.xml

99. Jung H, Jeon H-K, Kim H-J, Kim S-H. Immature Platelet Fraction: Establishment of a Reference Interval and Diagnostic Measure for Thrombocytopenia. Korean J Lab Med. 2010;30(5):451.

100. Seo A, Yuan D, Daniels S, Yuan S, Gallagher M, Wong ECC. Reference intervals for immature platelet fraction and immature platelet count. Int J Lab Hematol. 2015 Feb;37(1):e1–2.

101. Sukesh CN DD. Asymptomatic constitutional macrothrombovytopenia among west bengal blood donors. Am J Med. 2002;112(9):742–743.

102. Bhat R, Pai S. Immature platelet fraction: a significant platelet parameter in asymptomatic constitutional macrothrombocytopenia. Int J Lab Hematol. 2016 Jun 1;38(3):e45–7.

103. Rai S, Naik R, Pai M, Sinha R, Muhury M, Mathai A. Megakaryocytic alterations in thrombocytopenia: A bone marrow aspiration study. Indian J Pathol Microbiol. 2009;52(4):490.

104. Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood. 2010;115(2):168–186.

105. Naina HVK, Nair SC, Harris S, Woodfield G, Rees MI. Harris syndrome–a geographic perspective. J Thromb Haemost. 2005;3(11):2581–2582.

106. Ingram M, Coopersmith A. Reticulated platelets following acute blood loss. Br J Haematol. 1969 Sep;17(3):225–9.

107. Thon JN, Italiano JE. Does size matter in platelet production? Blood. 2012 Aug 23;120(8):1552–61.

108. Olsson R, Remberger M, Schaffer M, Berggren DM, Svahn BM, Mattsson J, et al. Graft failure in the modern era of allogeneic hematopoietic SCT. Bone Marrow Transplant. 2013;48(4):537–543.

ANNEXURE 1

IRB APPROVAL LETTER

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

March 23, 2015

Dr. Aswathy Ashok Menon
PG Registrar
Department of General Pathology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research Grant Project:**
Characterization of immature platelet fraction (IPF) in patients with thrombocytopenia presenting to a tertiary care center in India.
Dr. Aswathy Ashok Menon, General Pathology, Dr. Joy John Mammen, Transfusion Medicine and Immunohematology, Dr. Biju George, Haematology, Dr. Arpana Palle, Dr. Sukesh C. Nair, Transfusion Medicine and Immunohematology, Dr. Prasanna Samuel, Biostatistics, Ms. Josephine S, Transfusion Medicine, CMC, Vellore.

Ref: IRB Min No: 9345 [OBSERVE] dated 03.03.2015

Dear Dr. Aswathy Ashok Menon,

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

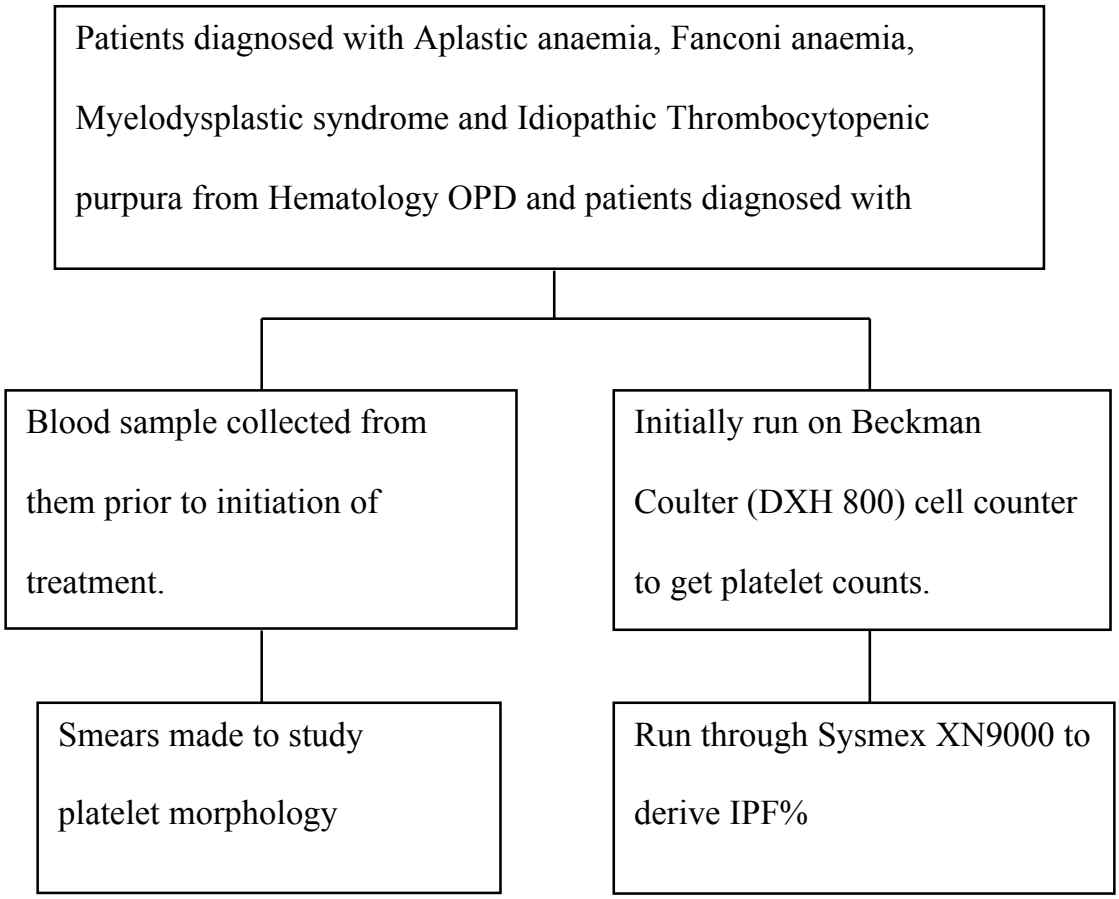
Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin) FRCP(Glasg
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Joy John Mammen, Transfusion Medicine and Immunohematology, CMC, Vellore. 1 of 5

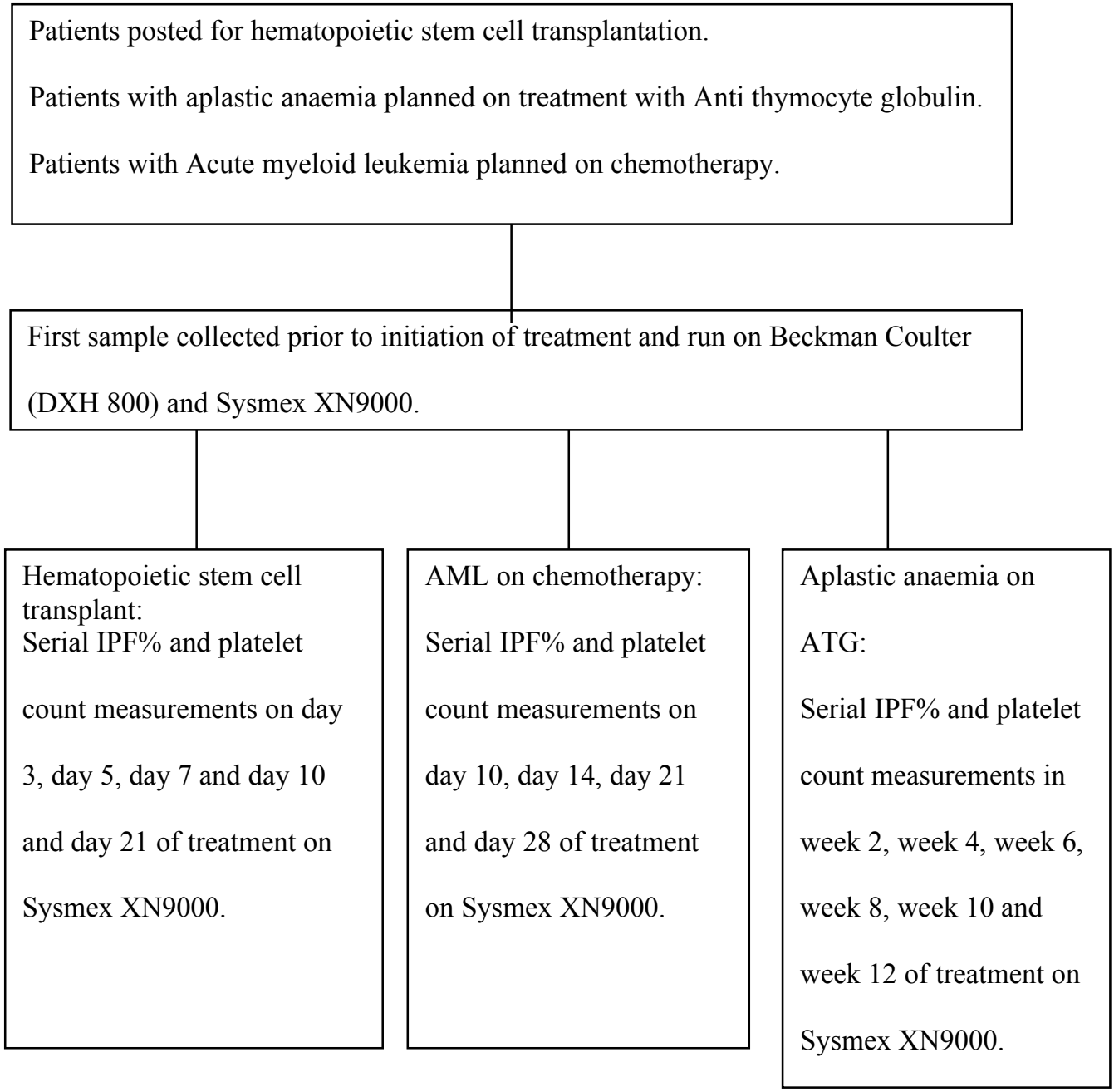
ANNEXURE 2

STUDY PROTOCOL

I. IPF in thrombocytopenia due to decreased production or peripheral destruction of platelets:



II. Trends of IPF in patients post hematopoietic stem cell transplantation:



ANNEXURE 3

DATA COLLECTION FORMS

IPF Study in Patients with Regenerating Bone Marrow

Study Number				**CATEGORY**	AA	CHEMO	BMT
Hospital Number		Name			Age		
Ward					Gender		
Category	**APLASTIC ON ATG**						
	Day 0	Week 2	Week 4	Week 6	Week8	Week 10	Week 12
Plt_1							
Plt_2							
IPF%							
Category	**CHEMO**						
	D0	D10	D14	D21	D28		
Plt_1							
Plt_2							
IPF%							
Category	**BMT**						
	D0	D1	D2	D3	D4	D5	Till day of recovery
Plt_1							
Plt_2							
IPF%							

Standard Data Collation form for Thrombocytopenic patients and controls

S. No	Hospital Number	Diagnosis	PLT-I	PLT-F	IPF	PDW	PCT	P-LCR	Megakary ocyte Adequacy
1									
2									
3									
4									
5									

S. No	Hospital Number	Diagnosis	PLT-S	Platelet size			Platelet cytoplasm			Total platelets assessed
1				Small	Large	Very large	Grey	Bluish grey	Blue	
2										
3										
4										
5										

ANNEXURE 4

STANDARD OPERATING PROTOCOL FOR UniCel DxH 800 SLIDE MAKER AND STAINER

2.0 Procedure to use UniCel DxH 800 Slide Maker and Stainer.

2.1 Purpose: To increase workflow efficiency and provide excellent smears every time and eventually increase the laboratories productivity.

2.2 Principle: The HaemaSphere technology captures the blood adherence to the aspiration tubing which provides a more reliable and consistent smear regardless of blood consistency.

2.3 Performance specification

140 slides/hour

2.4 Primary sample: K_2EDTA blood

2.5 Type of container: Lavender top vacutainer.

2.6 Sample and equipment requirement

1. Slide maker

a. 1 mL blood. b. Sample holding cassette. c. Beckman Coulter DxH 800 Slides d. Printer ribbon. e. Smear holding basket.

2. Stainer a. Modified Wrights stain. b. Modified Wright Giemsa stain. c. Wright stain buffer d. Distilled water.

2.7 Procedure

2. Open the slide ejector door and load the slides with the painted side up and the frosted side to the right side.

3. Close the slide ejector door.

4. Manually scan and fill in all the reagents and stains.

5. Ensure the communication status is glowing green (online).

6. Select the option make slide and stain on the “Detail status”.

7. Load the blood sample into the cassette provided by the company.

8. Then select/ check the processing mode whether it be the Primary or the secondary mode. Cassette is transfer to the barcode station where the barcodes are read and the cassettes are transferred to the left or right mixing stations where the samples are mixed by sixteen inversions. The primary mode is the fully automated mode where the analyser transports the sample and does the mixing and aspirates 100µl all of sample and processes it.

9. The Secondary mode or the emergency mode is used only to process emergency/STAT samples and samples with a low volume. On this mode the analyser stops a previous analysis and immediately smears the sample that has been fed.

10. Care should be taken that the bar coded samples are placed in a manner that they can be scanned by the analysers scanning system.

10. The clean spreader slide head on 22degree angle used. The slide is pushed from the smear shuttle into printer shuttle. the painted side print the barcode and transfer to the basket elevator for drying.

11. After the smear dries the robot arm picks the basket and sends it to the stainer where the slides get stained and dry.

12. After the smears get stained the robot arm takes the basket and puts them into the I/O drawer position.

Protocol options (DxH Slide maker and Stainer Default)

Bath	Supply type	Percentage	Duration
1	Modified Wright stain	100%	1 minute
2	Modified Wright Giemsa stain	100%	2 minutes
3	Stain/Buffer	Wright Giemsa 30%, Wright stain buffer 70%	6 minutes
4	Distilled H2O	100%	1min 30 sec
5	Distilled H2O	100%	2 minutes
Drying duration 3 minutes			

ANNEXURE 5

STANDARD OPERATING PROTOCOL FOR ENUMERATING PLATELETS ON A STAINED PERIPHERAL SMEAR

Estimation of Number of platelets:
It is very important to give an accurate estimation of the number of platelets as well as the description of the morphology. The abnormal platelets should describe such as giant or bizarre, platelet estimate can usually be made by each platelet in the average oil immersion field representing 15,000 platelets per mm3. It is important to remember that these estimates are only reliable when the blood has been drawn in EDTA and slides are prepared within an hour. This estimate should not be used when the smear has been made from a finger prick.

Platelets markedly decreased - 0 – 50,000/mm^3
Platelets moderately decreased - 51,000 – 1,00,000/mm^3
Platelets slightly adequate - 1,00,000 – 3,00,000/mm^3
Platelets slightly increased - 3,00,000 – 5,00,000/mm^3
Platelets moderately increased - 5,00,000 – 6,60,000/mm^3
Platelets markedly increased - More than 6,50,000/mm^3

QC procedures:
Duplicate checking

9.10 Calibration: NA

9.11 Interference: NA

9.12 Results: NA

9.13 Range of testing: Semi quantitative

9.14 Alert values: NA

9.15 Potential source of variation: NA

9.16 References:
1. Handbook of Medical Laboratory Technology, Second edition, Robert H Carman, 91-95.

ANNEXURE 6

SL NO	ENG	PLT0	SIP0	PLT1	SIP1	PLT2	SIP2	PLT3	SIP3	PLT4	SIP4	PLT5	SIP5	PLT6	SIP6	PLT7	SIP7	PLT8	SIP8	PLT9	SIP9	PLT10	SIP10	PLT11	SIP11	PLT12	SIP12	PLT13	SIP13	PLT14	SIP14
1	13	17000	3.6	99000	2.3	80000	2	62000	1.6	29000	1.1	21000	1.6	15000	1.4	10000	2.3	36000	2.5	28000	2.1	21000	1.6	39000	2.8	40000	4	51000	4.5	61000	4.9
2	14	66000	5.8	64000	5.7	49000	4.8	42000	4.2	23000	4.7	21000	3.7	65000	1.3	46000	1.2	49000	2.4	16000	1.6	5000	1	25000	3.2	38000	4.9	52000	7.7	63000	7.1
3	12	11000	0.2					31000	1.5	22000	1.6	17000	1.3	18000	1.7	11000	1.6	21000	7.6	12000	5.2	28000	4.3	24000	5.5	27000	5.5	47000	5.6	77000	4.5
4	13			59000	5.5	51000	4.3	41000	5.4	35000	3.6	29000	5	22000	3.4	16000	3.9	12000	4.3	25000	5.5	15000	5.7	22000	5.8	22000	8	48000	10.2	62000	11.7
5	12			30000	25.9	23000	21.7	25000	14.4	17000	13.3	30000	7.9	20000	8.1	13000	8.2	25000	5	15000	6.8	22000	8.2	23000	10.2	31000	19.8	51000	19.3	68000	18.7
6	12	23000	1.1	59000	3.4	49000	2.7	38000	2.4	26000	2.4	16000	2	8000	0.9	2000	2.7	25000	1.2	16000	1.5	102000	1.3	68000	2.7	65000	4.5	84000	7.1	149000	5.1
7	9	20000	1.2	12000	3.1	15000	1.6			8000	0	22000	1.2	17000	0.8	31000	3	21000	2.9	22000	4.9	23000	8.4	31000		50000		80000		109000	6
8	11	5000	0.6	25000	1.1	7000	0.3	13000	1.2	13000	1.8	10000	2.1							22000	2.3	18000	1.6	17000	4.6	14000	7.1	38000	4.5	25000	8.5
9	11	67000	3.3	147000	1.8	113000	1.5	101000	1.9	70000	1.5	58000	1.4	42000	1.9	24000	1.8	14000	2.3	29000	4.8	34000	6.2	57000	7.5	96000	5				
10	11	26000	2	60000	4	48000	4	39000	2.8	28000	2.7	20000	3	12000	2.4	36000	1.5	24000	2.8	31000	1.3	22000	1.3	17000	2.5	24000	2.9	38000		39000	5.8
11	15	21000	0.6	98000	2.8	73000	2.9	57000	2.6	44000	2.4	28000	2.2	21000	1.9	15000	1.5	46000	1.7	27000	1.9	18000	1.5	19000	1.6	14000	2.3	42000	1.4	37000	2.2
12	25	216000	3.6	92000	8.1	60000	5.3	49000	4.6	27000	5.1	57000	1.8	53000	1	36000	0.6	23000	0.5	15000	3.4	6000	5.7			14000	1.4	20000	3.9	13000	6.4
13	11	9000	2.6	49000	14.5	29000	10.9	23000	9.1	17000	9.9	23000	3.7	16000	4	17000	3.8	14000	2.9	22000	5.7	21000	6.9	35000	7	42000	8.4	74000	7.6	139000	7.6
14	12	2000	6.7	126000	1.4	93000	1.2	79000	1.4	62000	1.1	40000	1.1	20000	1.5	4000	1.5	0	0	1000	9.7	24000	5.5	22000	11.8	34000	10.9	50000	10.7		
15	21	32000	0.6	24000	4.1			26000	2.5			6000	1.1	29000	1.1	17000	1	10000	1.1	6000	8.8	12000	12.2	20000	16.5	25000	16.5	37000	14.9	27000	15.1
16	25	14000	0.7	15000	3.3	4000	1.9	11000	3.1	9000	1.3	9000	3.2							12000	3.3	12000	2.1	12000	2.5	8000	3.5	15000	3	14000	5.3
17	12	188000	1.9	183000	2.3	165000	2.6			80000	2.6	50000	2	30000	2.7	17000	1.9	6000	1.4	2000	3.8	36000	3.4	25000	3.5	27000	4.8	40000		37000	12.5
18	13	180000	1.6	270000	2.7	225000	3	178000	2.9	149000	2.8	118000	3.4	85000		61000		33000		12000	3.3	20000	3.4	69000	2.4	79000	3.7	114000	3.5	151000	2.8
19	13	64000	1.8	147000	1.5	115000	1.5	88000	1.7	60000	1.6	44000	1.6	29000		7000	3.1	2000	1.1	18000	1.8	5000	4.2	34000	6.9	45000	3.5	60000	3.7	102000	3.3
20	15	102000	1.8	100000	2.3	75000		66000	2.4	40000	2.9	23000	2.7	10000	2.3	4000	3.2	4000	4.5	9000	2	12000	3.2	11000	3	14000	4.4	21000	5.2	27000	4.6
21	21	39000	7	50000	5.9	36000	6.6	25000	5	17000	6.7	10000		5000	5.2	2000	5.8	3000	5.5	3000	3	2000	2.1	2000	6	5000	6.7	6000	12.3	9000	10.1
22	11	196000	3.2	198000	3.4	165000	3.5			100000	3.8	68000	3.4	43000	4	21000	4.6	8000	3.8	24000	0.9	19000	2.1	23000	3.2	21000	1.9	50000	5.1	67000	5.4
23	15	62000	10.9	51000	9.6	38000	10			18000	13.3	11000	11.3	5000	10.9	3000	8	0	0	3000	1.4	2000		3000	13.2	17000	16.2	36000	13.1	56000	10.9
24	15	137000	2.3	152000	2.1	106000	2	78000	2.3	53000	3.7	29000	3.5	12000	3.5	38000	2.3			11000	1.1	58000	0.6	21000		16000	1.1	57000	2.2	47000	4.2
25	13	49000		64000	8.3	54000	10.2	52000	10.2	39000	7.9	26000	7.8	13000	7.1	14000	3.1	27000	0.9	18000		20000	3.1	33000	6.3	28000	6.7	21000	6.8	27000	6.6
26	13	131000	0.9	143000	1.7	122000	1.5	83000	1.7	54000	2	28000	2	13000	1.6	6000	0.5	3000	1.1	19000	0.6	8000	2.4	31000	1.1	19000	2.4	22000	4.9	50000	3.7
27	28	67000	5.8	70000	5.9	60000	5.6	50000	5.3	32000	5	14000		5000	3.7	20000	3.2	4000	0	27000	3.5	8000	2.9	6000	3.3	33000	1	19000	1.6	28000	0.8
28	11	66000	14.4	54000	16.6	37000	18.3	27000	16.2	24000	13.7	15000	15.7	12000	15.3	25000	9.9			25000	11.2	43000	17.9	75000	13.9						
29	13							27000	3.2	16000	5.8			17000	6.8	8000	7.9					16000	12.3	26000	14.5			49000	10.6		
30	11	104000	1.5			31000	2.3	27000	3.6	23000	2.9	15000	4.2	13000	3.3	8000	5.3	14000	3.2	21000	4.4	27000	8.8	41000	10.5						
31	12	88000	4.6	84000	4.9			63000	4	58000	3.4			33000	3.4	19000	3.9	12000	5.3	14000	9			29000	12.4	42000	10.6				
32	14	55000	3.8			45000	3.1	44000	3.2	38000	4.3	30000	5.3	19000	7.7	13000	9.4	6000	6	4000	5.5	4000	8.3	10000	9.9	38000	7	45000	8.9	57000	9
33	19	53000	2.2	44000	3.3	31000	4.7	27000	5.6	20000	6.4							3000	6	8000	5.8	18000	7.3								
34	12					113000	2.6			93000	2.5			58000	2.7	43000	3.5	19000	4.5	52000	6.1					239000	6.5				
35	10					14000	14.9			20000	12	16000	10.9	17000	9.9	19000	9.3	23000	9.7	23000	13.1	47000	9.5								
36	13	59000	2.3	48000	3.3	27000	3.4	21000	3.9	16000	3.8	17000	3.5	19000	3.9	11000	5.1	23000	3.9	9000	3.7	19000	5.1	27000	8	31000	10	40000	9.7		
37	16	43000	3	37000	3.2	27000	3.4	23000	2.6	13000	2.1	7000	2.1	8000	1.9	12000	1.1	11000	1.4	12000	2.2	11000	2.1			10000	4.2	21000	5.7	25000	8
38	13	96000	1.6	50000	1.8			33000	1.6			24000	1.6			15000	1.7	9000	0.5	9000	1.8					19000	4.1				
39	14	84000	1	75000	1	63000	1.1	51000	1.1			15000	1.7			26000	0.6	41000	0.9	29000	1	36000	2.2	25000	1.8	45000	1.6	33000	3.3	27000	3
40	21	73000	4.7	57000	4.5	32000	3.6	23000	3	15000	3	9000	3.6	8000	3.4	24000	1.4	12000	2.5	11000	5.5	9000	5.5	5000	9.9					13000	11.1
41	0			4000	3.6	1000	4.7	12000	3.4	9000	1.3	1000	4.2	10000	1.5	19000	0.6	41000	1.4	16000	0.7	17000	0.7	20000	0.7	32000	0.9	14000	0.8	22000	0.4
42	0	1000	3.3	35000	0.7	6000	1.2	2000	4.3	3000	2.9	0	0	0	0			6000	2.1	0	0	2000	0	8000	3.1	1000	1.4	3000	2.9	21000	1.7
43	0	18000	1.4	34000	1.8	19000	2.3	12000	2.4	5000	2	3000	2.7	1000	1.3	11000	0.7	9000	0.1	14000	0.7	9000	0	8000	0.9	4000	1	6000	1.5	6000	0.7

PLT15	SIP15	PLT16	SIP16	PLT17	SIP17	PLT18	SIP18	PLT19	SIP19	PLT20	SIP20	PLT21	SIP21	PLT22	SIP22	PLT23	SIP23	PLT24	SIP24	PLT25	SIP25	PLT26	SIP26	PLT27	SIP27	PLT28	SIP28	PLT29	SIP29
76000	4.4																												
63000	9.3	80000	9.9																										
111000	2.8																												
72000	10.1	81000	9																										
112000	14.7	140000	16.2																										
180000	4.1	246000	3.7																										
29000	9.4	44000	9.6	26000	13.7	31000	14.2																						
59000	4.7																												
37000	2.8	35000																											
16000	5.6	13000	6.6	18000	5.3	7000	0.5	4000	12			5000	6.9	15000	5.8	23000	4.8	17000	6.5	15000	9.5	14000	7.8	15000	10.9	22000	11.7		
15000	14.5	10000	23.3	29000	11	11000	14	28000	13.9	31000	17.2	40000	17.8																
26000	3.3			21000	5	14000	8.6	15000	10	16000	9.3	24000	7			22000	12.2	24000	13.6	23000	13.6								
58000	6.8			101000	7.3							95000	5.5																
190000	2.6	224000		215000																									
150000	2.5	190000	2.5																										
		65000	7.6																										
15000	12	21000	10.4	35000	9.5			41000	10.6			41000	10.6																
98000	4.4			159000																									
82000	7.5	84000	6.5	70000	6.2	76000		82000																					
43000	5.6	47000		37000	7.4			46000	4	39000	8.1																		
32000	5.2	33000	7.4					68000																					
95000		91000																											
9000	2.6	5000	6.3	3000	11.4	6000	9.1	19000	3.1	8000	8.4			5000	11.1			12000	6.3			25000	6.4			26000	10.2		
26000	22.1					32000	20.6	82000	13																				
36000	7.3																												
7000	33.2	22000	15.4	15000	20.3			24000		19000	20.8	21000	13.6																
8000	0.6	22000	1.1	7000	0.8	2000	0.9	19000	0.2	19000	0.2	2000	0.2	4000	0	3000	0	37000	4	29000	1.5	50000	0.4	6000	0.5			6000	0.2
7000	2.6			16000	2.7	43000	1.3	22000	2.3	14000	1.7			10000	3.8	15000	4.6	20000	2.4	39000	0.1	6000	3.3	6000	6.2	6000	2.2	5000	4.1
8000	1	8000	2.9	6000	6	3000	2.4	6000	4.5																				

SL NO	AGE	SEX	DIAGNOSIS	PLTI	PLTF	PLTS	MPV	PDW	PCT	PLCR	IPF	MEG T	MEG A	SMALL	LARGE	CYTOPLASM
1	26	F	HYPERSPLENISM	41000	42000	45000	8.9				9.9	5	5	26	24	3
2	56	M	MDS (Adequate)	25000	15000	21500	9.9				13.6	1	6	22	28	3
3	53	M	MPN (Adequate)	51000	40000	51750	10.5				8	5	5	43	7	1
4	65	M	LYMPHOMA	22000	6000	10500	9.5	20.3	0.01	58.5	12.8	3	3	35	15	1
5	12	M	APLASTIC ANAEMIA	3000	1000	3000	10.9				8.1	1	1	9	1	1
6	75	F	MEGALOBLASTIC ANA	5000	2000	8250	9.9				8.3	3	3	22	3	1
7	62	M	MYELOMA	37000	40000	23250	12.8				12.2	5	6	23	27	3
8	48	M	ITP	23000	23000	21000	8.5	19.9	0.03	57.8	23.7	1	1	11	39	3
9	7	M	LEUKEMIA	14000	9000	6750	11	14.6	0.02	36.2	4.6	4	4	8	2	1
10	55	F	ITP	43000	42000	41250	8.5				14	1	1	29	20	5
11	8	F	ITP	20000	16000	10500	9.1				17.2	1	5	28	20	3
12	20	F	HYPERSPLENISM	91000	80000	55500	8.1				7.9	5	5	45	4	2
13	10	M	LEUKEMIA	27000	18000	9750	9.5				3.3	4	4	40	10	1
14	25	F	APLASTIC ANAEMIA	35000	22000	25500	11.3	14.4	0.04	44.4	4	3	3	39	11	1
15	33	M	HYPERSPLENISM	83000	115000	93000	4.5				17	5	5	9	41	2
16	35	F	ITP	6000	18000	15750	8.8				42.5	1	1	8	20	3
17	10	F	APLASTIC ANAEMIA	38000	33000	21750	9.6	24.1	0.07	52.7	3.9	3	3	44	6	1
18	16	F	MDS/MPN (Decreased)	7000	7000	11250	10.6	11.6	0.01	41.7	9.5	3	1	40	10	1
19	30	F	OTHERS - CKD, SICKLE	72000	66000	74250		18	0.09	51.8	12.8	5	5	26	24	1
20	15	M	HYPERSPLENISM	47000	44000	100500	11.8				15.5	5	5	34	16	3
21	55	F	MDS (Decreased)	24000	18000	36000	8.4				16	3	3	19	31	3
22	26	M	PNH	30000	17000	2250	10.6	20.8	0.04	45.8	5.9	3	3	5	0	1
23	33	M	APLASTIC ANAEMIA	5000	2000	5000	12				7.2	4	4		0	6
24	47	M	OTHERS - CKD	94000	93000	93000	8.9	23.2	0.13	54.9	17.4	5	5	13	37	3
25	18	F	ITP	7000	1000	3750	8.1				20.8	5	1	3	8	3
26	49	M	APLASTIC ANAEMIA	36000	33000	36750	8.8				7.3	3	4	46	4	1
27	71	M	APLASTIC ANAEMIA	92000	88000	63000	9.3	12.2	0.11	37.1	5.4	3	3	45	5	1
28	10	M	PNH	14000	7000	3000	8.3				13.6	2	1	8	17	3
29	9	M	APLASTIC ANAEMIA	44000	42000	53250		11.6	0.05	32	4.9	3	3	28	22	1
30	21	F	ITP	1000	2000	1500	8.9				25.1	1	2	1	3	3
31	52	F	APLASTIC ANAEMIA	4000	2000	1500	10				6.6	4	4	4	1	1
32	24	F	HYPERSPLENISM	85000	70000	80250	11.4				10.3	5	5	40	10	1
33	5	M	OSTEOPETROSIS	46000	37000	78000	13.5	13.4	0.04	34.6	5.5	5	5	33	17	1
34	9	F	ITP	2000	1000	1500	8.9				7.4	1	2	4	1	1
35	45	F	ITP - SECONDARY TO S	49000	42000	49500		15.2	0.05	36.2	5.3	1	1	39	11	1
36	8	F	APLASTIC ANAEMIA	1000	25000	19500	9	10.2	0.03	23.9	1.2	4	4	46	4	1
37	63	M	MDS (Adequate)	73000	63000	94500		13.7	0.08	38.5	8.8	1	1	14	36	1
38	5	M	APLASTIC ANAEMIA	36000	16000	12750					6	6	4	42	8	1
39	40	F	LEUKEMIA	23000	12000	12750					7	4	4	35	15	1
40	32	F	ITP	18000	16000	16500	11.7				9.8	1	2	13	36	3
41	40	M	HYPERSPLENISM	19000	22000	20250	12.5	16.8	0.05	46.5	13	5	5	16	34	3
42	63	M	CHRONIC ITP	54000	49000	36000		15.8	0.07	42.9	10.6	5	5	14	36	2
43	25	M	ITP	22000	22000	24000					37.4	1	2	8	36	3
44	22	F	ITP	12000	10000	5250					21.5	1	1	9	12	3
45	25	F	ITP	80000	48000	45000					10.1	5	5	10	39	3
46	52	F	MYELOMA	88000	81000	47250	12.7				5.5	3	6	27	23	1
47	48	M	CHRONIC ITP	36000	34000	18750	11	16.5	0.05	44.6	9.3	5	5	15	10	1
48	47	F	HYPERSPLENISM	52000	49000	46500	10.7	14.3	0.06	39.8	5.9	5	5	33	17	1
49	0	F	APLASTIC ANAEMIA	13000	19000	11250					20.5	1	1	27	23	3

50	47	M	CEMTP	45000	87000	78000	9.7				58.9	5	5	33	61	4
51	13	M	LEUKEMIA	7000	7000	3750	9.8	15.5	0.01	37.4	18.5	4	4	4	1	3
52	62	F	INFECTION	39000	38000	21000	9	15.9	0.05	46.3	12.6	1	1	45	5	3
53	62	F	CHRONIC ITP	71000	65000	36000	7.2	14.6	0.09	37.3	4.4	1	1	48	2	1
54	6	M	HYPERSPLENISM	80000	76000	47250	9.8	10.3	0.1	31.9	2.6	5	5	49	1	1
55	61	M	MDS (Adequate)	7000	1000	5000					15.4	2	2			
56	18	F	MEGALOBLASTIC ANA	30000	35000	25500	14.9				12.2	5	5	43	7	3
57	36	M	CEMTP	47000	57000	62250	8.1				39.9	5	5	36	13	3
58	2	M	INFECTION - DENGUE	19000	15000	15000	9.8				7.9	5	5	3	1	1
59	71	M	APLASTIC ANAEMIA	9000	12000	4500	10.8				11.2	4	3	4	1	3
60	59	M	CEMTP	21000	78000	81000	10.4				46.8	5	5	21	28	3
61	35	M	HYPERSPLENISM	90000	87000	54000	10.7	18.7	0.13	50.2	6.7	5	5	49	1	1
62	37	M	CHRONIC ITP	81000	79000	50250	9.9	17.5	0.12	52.6	14.6	1	1	27	15	2
63	9	F	CHRONIC ITP	6000	4000	6750	8.2				41.8	2	2	1	8	3
64	58	M	APLASTIC ANAEMIA	12000	9000	3000	9.8	18.2	0.01	38.6	8.6	5	4	4	1	1
65	34	M	HYPERSPLENISM	30000	59000	38250	8.9				44.8	5	5	10	40	2
66	61	M	INFECTION	37000	36000	28500	14.7	17.5	0.04	39.9	5.6	5	5	47	3	3
67	19	M	CEMTP	45000	60000	62250	11.6				57.2	5	5	12	34	3
68	45	F	ITP	12000	32000	10500	8.8				26.8	1	1	29	19	4
69	38	M	APLASTIC ANAEMIA	17000	17000	15750	7.5	16	0.02	41.5	9.5	3	6	46	4	3
70	36	M	LEUKEMIA	16000	10000	12750	8.8	13.5	0.02	38	4.7	4	4	19	1	1
71	18	M	APLASTIC ANAEMIA	10000	6000	7500	10.6				4	4	6	19	1	2
72	24	F	MDS(Decreased)	8000	4000	3000	10.9				20.5	3	1	7	3	2
73	9	F	HYPERSPLENISM	56000	89000	74250	10.2				16.3	5	5	67	31	2
74	39	M	ITP	37000	44000	28250					17.2	5	1	37	13	3
75	38	M	CEMTP	47000	59000	67500	15.9				48.6	5	5	19	29	3
76	46	M	CEMTP	62000	78000	69000	7				48.5	5	5	46	52	3
77	14	M	LEUKEMIA	80000	79000	69750	11.6	12.1	0.1	32.1	3.2	1	1	48	2	3
78	30	F	CEMTP	54000	66000	108750	12.6				48.8	5	5	20	30	3
79	32	F	CEMTP	55000	63000	54750					37.9	5	5	20	29	4
80	40	M	CEMTP	17000	77000	45000	8.7				50.7	5	5	14	36	3
81	1	M	ITP	54000	51000	23250	9.7	16.6	0.09	45.7	9.2	1	2	22	60	4
82	64	F	PNH	9000	9000	6750	7.8				17.5	3	3	43	7	3
83	35	M	APLASTIC ANAEMIA	12000	7000	11250	10.6	16.7	0.01	39.2	5.5	4	3	17	2	3
84	2	F	ITP	11000	10000	7500	10				23.5	5	1	18	2	3
85	45	M	APLASTIC ANAEMIA	13000	10000	7500	9.4				4.9	1	3	15	9	3
86	45	M	APLASTIC ANAEMIA	12000	7000	3750	9.7				7	3	3	18	2	3
87	32	M	CHRONIC ITP	5000	4000	3750	8.1				30.1	2	2	7	3	2
88	16	M	APLASTIC ANAEMIA	33000	30000	20250	9.3	11.9	0.03	32.1	7.3	3	3	6	4	3
89	16	F	HYPERSPLENISM	48000	57000	50250	8.5				15.1	5	5	42	8	3
90	35	F	APLASTIC ANAEMIA	18000	14000	8250	11	13.1	0.02	44.9	5.7	1	3	26	23	3
91	3	M	CHRONIC ITP	9000	7000	3000	11.3				20.1	5	5	19	1	2
92	27	M	MDS (Adequate)	42000	36000	33000	11.3				14.2	1	1	6	3	4
93	56	F	MPN (Decreased)	10000	10000	2250	8.2	12.4	0.03	39.3	17.4	3	3	32	18	3
94	11	F	APLASTIC ANAEMIA	30000	28000	19500	8	13.2	0.04	35.7	5.1	4	3	9	1	3
95	58	F	LYMPHOMA	16000	15000	15000	8	10.4	0.03	37.7	5.2	4	4	40	10	3
96	23	F	CHRONIC ITP	16000	8000	9750	11.5				38.9	2	2	45	5	3
97	51	F	APLASTIC ANAEMIA	20000	16000	11250	8.1	20.5	0.03	51.2	6.8	1	3	18	28	4
98	44	M	APLASTIC ANAEMIA	15000	15000	3750	10	15.3	0.02	42.7	6.1	3	3	45	5	3
99	60	M	MDS (Adequate)	29000	21000	16500	8.6	15.3	0.03	39.8	4.1	1	1	13	2	3

100	26	M	APLASTIC ANAEMIA	12000	7000	4500	9.4				5.2	4	4	24	1	3
101	34	M	APLASTIC ANAEMIA	8000	6000	7500	9.4				10.1	4	4	9	1	3
102	6	F	ITP	46000	45000	39750	6	15.5	0.07	41.1	8.7	1	1	23	2	3
103	0.5	M	WAS	18000	21000	11250	9.5				7.3	1	1	38	12	3
104	7	M	LEUKEMIA	18000	16000	15750	9.8				9.2	3	3	21	4	3
105	33	M	LEUKEMIA	30000	29000	12000	8.1	10.8	0.03	22.5	9.2	3	3	49	1	3
106	50	F	MDS (Decreased)	16000	14000	9000	9.2	12.3	0.02	37.4	3.7	3	3	26	4	3
107	42	M	HYPERSPLENISM	61000	61000	39750	11.2	11.6	0.08	43.2	10.2	1	1	10	0	2
108	4	M	HYPERSPLENISM	16000	20000	24750	9				38	1	1	25	25	5
109	26	F	MDS (Adequate)	17000	14000	10500					14.1	1	2	27	23	4
110	42	M	HYPERSPLENISM	1000	1000	0	8.2				29.5	2	1	35	15	3
111	11	M	LEUKEMIA	51000	32000	3750	6.8				3.4	4	4		0	3
112	60	M	MEGALOBLASTIC ANA	26000	20000	21750	10.7				8.7	5	5	20	0	3
113	30	F	ITP	59000	49000	59250	12	16.8	0.09	45	6	5	5	44	6	3
114	56	F	CHRONIC ITP	9000	12000	13500	8.4				36.7	5	5	45	5	3
115	14	F	APLASTIC ANAEMIA	9000	7000	2250	6.3	10	0.01	17.4	1.9	3	3	26	23	4
116	22	M	MPN (Adequate)	10000	4000	750	11.7	8.5	0.02	24.4	27.2	5	5	5	0	3
117	5	M	LEUKEMIA	11000	13000	4500	9.5				3.5	4	4	2	0	1
118	11	M	APLASTIC ANAEMIA	38000	30000	18000	10.1				6.2	4	6	5	0	1
119	5	M	LEUKEMIA	94000	77000	69750	12.2	17.2	0.11	40.2	8.6	4	4	45	5	3
120	13	F	CHRONIC ITP	33000	35000	34500	12				18.2	5	5	44	6	3
121	24	F	LEUKEMIA	13000	9000	5250	27.8	10.2	0.02	32.8	6.1	4	3	19	31	3
122	49	M	APLASTIC ANAEMIA	12000	9000	4500	16.9				8.6	5	5	10	0	3
123	70	F	APLASTIC ANAEMIA	3000	3000	9750	8.5				5.2	3	4	9	1	3
124	43	M	LEUKEMIA	64000	54000	61500	8.4	15.5	0.07	33	4.5	3	1	13	2	3
125	34	M	LEUKEMIA	58000	61000	51000		16.3	0.08	41.5	8.2	4	4	49	1	3
126	59	F	CEMTP	70000	55000	56250	10.3				34.8	5	5	40	10	3
127	68	F	APLASTIC ANAEMIA	3000	1000	21000	13.8				9.4	4	6	44	52	3
128	18	F	APLASTIC ANAEMIA	11000	5000	5250	12.3				11.3	3	6	25	0	3
129	25	F	ITP	16000	15000	31500	13.1				28.9	1	2	5	0	3
130	52	M	HYPERSPLENISM	42000	42000	41250	10.1	23	0.07	64.8	19.2	1	1	45	5	3
131	5	M	MDS (decreased)	45000	47000	45750	12.6	19.1	0.07	46.1	11.2	7	1	33	16	3
132	9	M	ITP	59000	75000	78000	6.8				38.7	5	5	44	6	3
133	10	M	LEUKEMIA	17000	16000	8250	11.2	10.1	0.02	23.6	5.1	4	4	51	51	3
134	5	M	ITP	8000	7000	3750	10.5				17	5	5	10	0	3
135	21	M	MEGALOBLASTIC ANA	62000	32000	22500	8.3				7.7	1	3	2	2	3
136	28	M	APLASTIC ANAEMIA	49000	47000	39000		11.2	0.06	33.4	3	6	6	46	4	3
137	30	F	ITP	9000	3000	17250	10.4				27.8	5	2	50	0	6
138	76	M	CHRONIC ITP	74000	63000	74250	8.9				22.4	5	5	42	8	3
139	8	M	APLASTIC ANAEMIA	19000	16000	4500	8.3				1.6	5	5	83	17	2
140	59	M	OTHERS - DRUG INDUC	22000	23000	15750	9.5	14.2	0.03	42.2	3.5	3	1	10	0	2
141	6	F	APLASTIC ANAEMIA	25000	23000	12750	9.2				8.1	4	4	25	0	2
142	35	F	CHRONIC ITP	7000	11000	0	13.7				28.7	1	2	18	2	2
143	52	M	OTHERS - CKD	96000	88000		8.7	13.9	0.14	57.1	11.3	3	1	1	9	2
144	9	F	APLASTIC ANAEMIA	62000	60000	51750	8.9	11.5	0.08	35.9	5.5	1	6	40	10	3
145	44	M	HYPERSPLENISM	37000	30000	23250	8.6				6.2	1	1	40	10	2
146	29	M	APLASTIC ANAEMIA	4000	2000	1500	8.7				5.5	4	4	50	0	2
147	41	M	LEUKEMIA	69000	63000	59250	12.2	11.5	0.09	35.6	4.7	1	1	2	0	2
148	63	M	APLASTIC ANAEMIA	3000	2000	0	11				4	4	4	70	10	3
149	41	F	APLASTIC ANAEMIA	3000	2000	0	8				2.4	4	4		0	6

150	61	M	MEGALOBLASTIC ANA	15000	12000	9000	11.9				5.4	1	1		0	6
151	44	M	APLASTIC ANAEMIA	4000	3000	3000	10.8	11.1	0.01	39.4	7.2	3	3	10	0	1
152	23	F	APLASTIC ANAEMIA	5000	1000	2250	14.3				15.4	3	3	5	0	2
153	40	F	CEMTP	48000	82000	89250					46.1	5	5	5	0	3
154	1	F	ITP	57000	50000	27750	3.9				15.1	5	1	54	43	3
155	31	M	MPN (Decreased)	5000	10000	7500	10.8				29.2	3	3	30	20	3
156	14	F	LEUKEMIA	6000	3000	0	9				13.3	5	5	3	7	3
157	60	F	LEUKEMIA	81000	85000	75000	10.8	15.6	0.12	43.8	4.6	3	1		0	6
158	36	M	HYPERSPLENISM	27000	61000	62250	12.2				34.1	5	5	44	6	2
159	29	F	ITP	58000	57000	42000	11				14.1	5	5	50	50	3
160	30	F	HYPERSPLENISM	17000	17000	22500	9.3				10	1	1	46	4	3
161	4	F	OTHERS - KMS	7000	3000	6750	11				25.3	1	1	43	7	3
162	66	F	MDS (decreased)	39000	36000	30000	8.6				19.8	7	8	45	5	3
163	22	F	APLASTIC ANAEMIA	8000	22000	6000	16.4	13.7	0.03	30.7	2.6	3	3	5	3	3
164	61	F	CEMTP	57000	69000	64500	10.5				36.3	1	1	41	9	1
165	24	F	APLASTIC ANAEMIA	41000	34000	24750	10.2				8.6	1	1	10	0	1
166	31	F	CHRONIC ITP	24000	15000	13500					9.9	2	2	92	20	4
167	22	F	HYPERSPLENISM	92000	79000	60000	11.4				8.3	5	5	23	2	3
168	20	M	ITP	47000	79000	22500	7.8	11.9	0.09	26.3	1.9	5	5	21	4	3
169	54	M	LYMPHOMA	81000	2000	40500	10.4	4.9	0	24.1	2.6	1	1	41	9	6
170	7	M	ITP	17000	22000	21000	10.9				10	1	2	46	3	3
171	11	F	CHRONIC ITP	6000	2000	5250	10.2				40.4	1	1	50		3
172	10	M	APLASTIC ANAEMIA	9000	5000	9000					3.9	3	4	43	7	3
173	59	M	MYELOMA	78000	72000	75000	15				12.7	3	3	7	3	3
174	11	F	ITP	97000	98000	89250	10.4				17.3	1	2	10	0	3
175	34	F	PNH	52000	44000	63000		13.1	0.06	35.9	4.6	1	1	46	4	3
176	54	F	APLASTIC ANAEMIA	1000	20000	21000	10	8.4	0.02	18.8	0.8	4	4	72	26	3
177	50	M	LYMPHOMA	3000	1000	750	11.7	5.9	0	15.2	5.3	1	1	48	2	3
178	38	F	ITP	26000	14000	20250	11.1	17	0.03	41.6	15.2	1	2	30	0	3
179	36	F	MDS (Decreased)	38000	21000	34500	12.1				17.7	7	4		0	6
180	14	F	APLASTIC ANAEMIA	9000	2000	0	8				7.6	4	4	30	20	3
181	20	M	ITP	18000	11000	5250	10.5				14.4	1	1	44	6	3
182	15	F	LEUKEMIA	18000	16000	9750	8.5				15.1	3	4		0	6
183	38	M	APLASTIC ANAEMIA	32000	19000	20250	10.1				6.9	1	3	49	1	3
184	51	F	MEGALOBLASTIC ANA	68000	54000	84000	13.6				7.8	5	5	13	6	3
185	57	F	OTHERS - BSS	10000	17000	70500	13.9				63.9	1	1	23	6	3
186	59	F	CHRONIC ITP	19000	25000	32250	10.6				28.8	5	5	50	0	3
187	4	F	LEUKEMIA	11000	10000	7500	10.8				12.8	8	6	28	2	3
188	49	F	CHRONIC ITP	7000	8000	12000					43.6	5	5	50	0	3
189	17	F	OTHERS - BSS	14000	22000	26250	9.5				60.6	5	5	29	67	3
190	44	F	MDS (decreased)	17000	13000	10500	14				13.7	8	6	40	10	3
191	29	F	ITP	30000	19000	24750	11.7				15.5	1	1	10		3
192	39	M	CHRONIC ITP	32000	34000	24000	9	17.7	0.05	55.9	11.6	1	2	28	21	3
193	14	F	MDS (decreased)	16000	14000	15750	7.7				16.1	8	8	21	28	3
194	49	F	APLASTIC ANAEMIA	6000	1000	2250	8.5	11	0	36.2	10.1	4	4	19	11	2
195	46	F	MPN (Decreasd)	32000	26000	40500	7.8	12.5	0.04	30.6	5.2	8	8	46	4	3
196	22	M	LEUKEMIA	34000	27000	37500	7.8	16.4	0.04	37	3.1	4	4	42	8	3